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DNA encoding interleukin-1 receptor antagonist (IL-1ra beta)

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CORE TERMS: polypeptide, beta, sequence, polynucleotide, cell, protein, nucleotide, fragment, disease, amino acid, receptor, region, host, gene, encoding, isolated, compound, variant, antagonist, antibody, assay, residue, binding, peptide, alpha, cdna, inflammatory, agonist, vector, screening

ABST:
IL-1ra beta polypeptides and polynucleotides and methods for producing such polypeptides by recombinant techniques are disclosed. Also disclosed are methods for utilizing IL-1ra beta polypeptides and polynucleotides in the design of protocols for the treatment of chronic and acute inflammation, septicemia, arthritis, inflammatory bowel disease, graft vs. host disease, autoimmunity, stroke, cardiac ischemia, acute respiratory disease syndrome (ARDS), psoriasis, restenosis, traumatic brain injury, AIDS, cachexia., among others, and diagnostic assays for such conditions.

NO-OF-CLAIMS: 19

EXMPL-CLAIM: 1

NO-OF-FIGURES: 1

NO-DRWNG-PP: 1

SUM:

FIELD OF INVENTION

This invention relates to newly identified polynucleotides, polypeptides encoded by them and to the use of such polynucleotides and polypeptides, and to their production. More particularly, the polynucleotides and polypeptides of the present invention relate to Interleukin-1 family, hereinafter referred to as IL-1ra beta. The invention also relates to inhibiting or activating the action of such polynucleotides and polypeptides.

BACKGROUND OF THE INVENTION

Interleukin 1 refers to two proteins (IL1 alpha and IL1 beta) which play a key role early in the inflammatory response [for a review see C. A. Dinarello, Blood, 87:2095-2147 (1996) and references therein]. Both proteins are made as 31 kDal intracellular precursor proteins which are cleaved upon secretion to yield mature carboxy-terminal 17 kDal fragments which are biologically active. In the case of IL-1 beta is active, this cleavage involves an intracellular cysteine protease, known as ICE, which is required to release the active fragment from the inactive precursor. The precursor of IL-1 alpha is active.

These two proteins act by binding to cell surface receptors found on almost all cell types and triggering a range of responses either alone or in concert with other secreted factors. These range from effects on proliferation (eg of fibroblasts, T cells), apoptosis (eg A375 melanoma cells), cytokine induction (eg of TNF, IL1, IL8), receptor activation (eg E-selectin), eicosanoid production (eg PGE2) and the secretion of degradative enzymes (eg collagenase). To achieve this, IL-1 activates transcription factors such as NF- kappa B and AP-1, Several of the activities of IL-1 action on target cells are believed to be mediated through activation of kinase cascades that have also been associated with cellular stresses, such as the stress activated MAP kinases JNK/SAPK and p38.

A third member of the IL-1 family was subsequently discovered which acts as a natural antagonist of IL-1 alpha and IL-1 beta by binding to the IL-1 receptor but not transducing an intracellular signal or a biological response. The protein was called IL-1ra (for IL-1 receptor antagonist) or IRAP (for IL-1 receptor antagonist protein). At least three alternatively splice forms of IL-1ra exist: one encodes a secreted protein, and the other two encode intracellular proteins. The relative role of the three forms and reason for their different localization is not known. All three proteins, IL-1 alpha , IL-1 beta and IL-1ra share approximately 25-30% amino acid identity and a similar three-dimensional structure consisting of twelve beta - strands folded into a beta -barrel, with an internal thrice repeated structural motif.

There are three known IL-1 receptor subunits. The active receptor complex consists of the type I receptor and IL1RAcP (for IL-1 accessory protein). The type I receptor is responsible for binding of the three ligands, and is able to do so in the absence of the IL1RAcP. However signal transduction requires interaction of IL-1 alpha or beta with the IL1RAcP. IL-1ra does not interact with the IL-1 RAcP and hence cannot signal. A third receptor subunit, the type II receptor, binds IL-1 alpha and IL-1 beta but cannot signal due to its lack of an intracellular domain. Rather it act as a decoy either in its membrane form or an antagonist in a cleaved secreted form, and hence inhibits IL-1 activity. It only weakly binds IL-1ra.

Many studies using IL-1ra, soluble IL-1 R, derived from the intracellular domain of the type I IL-1R, antibodies to IL-1 alpha or beta , and transgenic knockouts of these genes have shown conclusively that the IL-1 s play a key role in a number of pathophysiologies (see C. A. Dinarello, Blood 87:2095-2147 (1996) for a review). For example, IL-1ra has been shown to be effective in animal models of septic shock, rheumatoid arthritis, graft versus host disease, stroke, cardiac ischemia, and is currently in clinical trials for some of these indications. Moreover, IL-1 alpha and beta have shown some potential as hematopoietic stem cell stimulators with potential as radio and chemoprotectants.

More recently, a more distant member of the IL-1 family was identified. This protein, originally isolated through its ability to induce Interferon gamma in T cells and hence called Interferon gamma inducing factor (IGIF) [H. Okamura et al., Nature 378:88-91 (1995)], was subsequently shown to fold in a similar structure to the IL-1s and share weak amino acid identity [Bazan et al., Nature 379:59] (1996). The name IL-1 gamma was proposed. IGIF appears to play a direct role in the liver damage which occurs during toxic shock and is therefore like the other IL-1s in playing an early role in inflammatory and stressful conditions.

This indicates that these Interleukin-1s have an established, proven history as therapeutic targets. Clearly there is a need for identification and characterization of further members of Interleukin-1 family which can play a role in preventing, ameliorating or correcting dysfunctions or diseases, including, but not limited to, chronic and acute inflanmmation, septicenmia, shock, arthritis, inflammatory bowel disease, graft vs. host disease, autoimmunity, stroke, cardiac ischemia, acute respiratory disease syndrome (ARDS), psoriasis, restenosis, traumatic brain injury, AIDS, and cachexia.

SUMMARY OF THE INVENTION

In one aspect, the invention relates to IL-1ra beta polypeptides and recombinant materials and methods for their production. Another aspect of the invention relates to methods for using such IL-1ra beta polypeptides and polynucleotides. Such uses include the treatment of chronic and acute inflammation, septicemia, arthritis, inflammatory bowel disease, graft vs. host disease, autoimmunity, stroke, shock, cardiac ischemia, acute respiratory disease syndrome (ARDS), psoriasis, restenosis, traumatic brain injury, AIDS, cachexia, among others. In still another aspect, the invention relates to methods to identify agonists and

antagonists using the materials provided by the invention, and treating conditions associated with IL-1ra beta imbalance with the identified compounds. Yet another aspect of the invention relates to diagnostic assays for detecting diseases associated with inappropriate IL-1ra beta activity or levels.

DRWDESC:
BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows the nucleotide and deduced amino acid sequence of human IL-1ra beta. SEQ ID NOS: 1 and 2.

DETDESC:

DESCRIPTION OF THE INVENTION

Definitions

The following definitions are provided to facilitate understanding of certain terms used frequently herein.

"IL-1ra beta" refers generally to a polypeptide having the amino acid sequence set forth in SEQ ID NO: 2 or an allelic variant thereof.

"IL-1ra beta activity or IL-1ra beta polypeptide activity" or "biological activity of the IL-1ra beta or IL-1ra beta polypeptide" refers to the metabolic or physiologic function of said IL-1ra beta including similar activities or improved activities or these activities with decreased undesirable side-effects. Also included are antigenic and immunogenic activities of said IL-1ra beta.

"IL-1ra beta polypeptides" refers to polypeptides with amino acid sequences sufficiently similar to IL-1ra beta sequences, preferably exhibiting at least one biological activity of the IL-1ra beta.

"IL-1ra beta gene" refers to a polynucleotide having the nucleotide sequence set forth in SEQ ID NO: 1 or allelic variants thereof and/or their complements.

"IL-1ra beta polynucleotides" refers to polynucleotides containing a nucleotide sequence which encodes a IL-1ra beta polypeptide or fragment thereof, or a nucleotide sequence which has at least 80% identity to a nucleotide sequence encoding the polypeptide of SEQ ID NO:2 or the corresponding fragment thereof, or a nucleotide sequence which has sufficient 80% identity to a nucleotide sequence contained in SEQ ID NO:1 to hybridize under conditions useable for amplification or for use as a probe or marker.

"Antibodies" as used herein includes polyclonal and monoclonal antibodies, chimeric, single chain, and humanized antibodies, as well as Fab fragments, including the products of an Pab or other immunoglobulin expression library.

"Isolated" means altered "by the hand of man" from the natural state. If an "isolated" composition or substance occurs in nature, it has been changed or removed from its original environment, or both. For example, a polynucleotide or a polypeptide naturally present in a living animal is not "isolated," but the same polynucleotide or polypeptide separated from the coexisting materials of its natural state is "isolated", as the term is employed herein.

"Polynucleotide" generally refers to any polyribonucleotide or polydeoxribonucleotide, which may be unmodified RNA or DNA or modified RNA or DNA. "Polynucleotides" include, without

limitation single- and double-stranded DNA, DNA that is a mixture of single- and double-stranded regions, single- and double-stranded RNA, and RNA that is mixture of single- and double-stranded regions, hybrid molecules comprising DNA and RNA that may be single-stranded or, more typically, double-stranded or a mixture of single- and double-stranded regions. In addition, "polynucleotide" refers to triple-stranded regions comprising RNA or DNA or both RNA and DNA. The term polynucleotide also includes DNAs or RNAs containing one or more modified bases and DNAs or RNAs with backbones modified for stability or for other reasons. "Modified" bases include, for example, tritylated bases and unusual bases such as inosine. A variety of modifications has been made to DNA and RNA; thus, "polynucleotide" embraces chemically, enzymatically or metabolically modified forms of polynucleotides as typically found in nature, as well as the chemical forms of DNA and RNA characteristic of viruses and cells. "Polynucleotide" also embraces relatively short polynucleotides, often referred to as oligonucleotides.

"Polypeptide" refers to any peptide or protein comprising two or more amino acids joined to each other by peptide bonds or modified peptide bonds, i.e., peptide isosteres. "Polypeptide" refers to both short chains, commonly referred to as peptides, oligopeptides or oligomers, and to longer chains, generally referred to as proteins. Polypeptides may contain amino acids other than the 20 gene-encoded amino acids. "Polypeptides" include amino acid sequences modified either by natural processes, such as posttranslational processing, or by chemical modification techniques which are well known in the art. Such modifications are well described in basic texts and in more detailed monographs, as well as in a voluminous research literature. Modifications can occur anywhere in a polypeptide, including the peptide backbone, the amino acid side-chains and the amino or carboxyl termini. It will be appreciated that the same type of modification may be present in the same or varying degrees at several sites in a given polypeptide. Also, a given polypeptide may contain many types of modifications. Polypeptides may be branched as a result of ubiquitination, and they may be cyclic, with or without branching. Cyclic, branched and branched cyclic polypeptides may result from post-translation natural processes or may be made by synthetic methods. Modifications include acetylation, acylation, ADP-ribosylation, amidation, covalent attachment of flavin, covalent attachment of a heme moiety, covalent attachment of a nucleotide or nucleotide derivative, covalent attachment of a lipid or lipid derivative, covalent attachment of phosphotidylinositol, cross-linking, cyclization, disulfide bond formation, demethylation, formation of covalent cross-links, formation of cystine, formation of pyroglutamate, formylation, gamma-carboxylation, glycosylation, GPI anchor formation, hydroxylation, iodination, methylation, myristoylation, oxidation, proteolytic processing, phosphorylation, prenylation, racemization, selenoylation, sulfation, transfer-RNA mediated addition of amino acids to proteins such as arginylation, and ubiquitination. See, for instance, PROTEINS-STRUCTURE AND MOLECULAR PROPERTIES, 2nd Ed., T. E. Creighton, W. H. Freeman and Company, N.Y., 1993 and Wold, F., Posttranslational Protein Modifications: Perspectives and Prospects, pgs. 1-12 in POSTTRANSLATIONAL COVALENT MODIFICATION OF PROTEINS, B. C. Johnson, Ed., Academic Press, N.Y., 1983; Seifter et al., "Analysis for protein modifications and nonprotein cofactors", Meth Enzymol (1990) 182:626-646 and Rattan et al., "Protein Synthesis: Posttranslational Modifications and Aging", Ann NY Acad Sci (1992) 663:48-62.

"Variant" as the term is used herein, is a polynucleotide or polypeptide that differs from a reference polynucleotide or polypeptide respectively, but retains essential properties. A typical variant of a polynucleotide differs in nucleotide sequence from another, reference polynucleotide. Changes in the nucleotide sequence of the variant may or may not alter the amino acid sequence of a polypeptide encoded by the reference polynucleotide. Nucleotide changes may result in amino acid substitutions, additions, deletions, fusions and truncations in the polypeptide encoded by the reference sequence, as discussed below. A typical variant of a polypeptide differs in amino acid sequence from another, reference polypeptide. Generally, differences are limited so that the sequences of the reference polypeptide and the variant are closely similar overall and, in many regions, identical. A variant and reference

polypeptide may differ in amino acid sequence by one or more substitutions, additions, deletions in any combination. A substituted or inserted amino acid residue may or may not be one encoded by the genetic code. A variant of a polynucleotide or polypeptide may be a naturally occurring such as an allelic variant, or it may be a variant that is not known to occur naturally. Non-naturally occurring variants of polynucleotides and polypeptides may be made by mutagenesis techniques or by direct synthesis.

"Identity" is a measure of the identity of nucleotide sequences or amino acid sequences. In general, the sequences are aligned so that the highest order match is obtained. "Identity" per se has an art-recognized meaning and can be calculated using published techniques. See, e.g.: (COMPUTATIONAL MOLECULAR BIOLOGY; Lesk, A. M., ed., Oxford University Press, N.Y., 1988; BIOCOMPUTING: INFORMATICS AND GENOME PROJECTS, Smith, D. W., ed., Academic Press, N.Y., 1993; COMPUTER ANALYSIS OF SEQUENCE DATA, PART I, Griffin, A. M., and Griffin, H. G., eds., Humana Press, N.J., 1994; SEQUENCE ANALYSIS IN MOLECULAR BIOLOGY, von Heinje, G., Academic Press, 1987; and SEQUENCE ANALYSIS PRIMER, Gribskov, M. and Devereux, J., eds., M Stockton Press, N.Y., 1991). While there exist a number of methods to measure identity between two polynucleotide or polypeptide sequences, the term "identity" is well known to skilled artisans (Carillo, H., and Lipton, D., SIAM J Applied Math (1988) 48:1073). Methods commonly employed to determine identity or similarity between two sequences include, but are not limited to, those disclosed in Guide to Huge Computers, Martin J. Bishop, ed., Academic Press, San Diego, 1994, and Carillo, H., and Lipton, D., SIAM J Applied Math (1988) 48:1073. Methods to determine identity and similarity are codified in computer programs. Preferred computer program methods to determine identity and similarity between two sequences include, but are not limited to, GCS program package (Devereux, J., et al., Nucleic Acids Research (1984) 12 (1):387), BLASTP, BLASTN, FASTA (Atschul, S. F. et al., J Molec Biol (1990) 215:403).

Polypeptides of the Invention

The IL-1ra beta polypeptides of the present invention include the polypeptide of SEQ ID NO:2 (in particular the mature polypeptide) as well as IL-1ra beta polypeptides and which have at least 80% identity to the polypeptide of SEQ ID NO:2 or the relevant portion and more preferably at least 85% identity, and still more preferably at least 90% identity, and even still more preferably at least 95% identity to SEQ ID NO:2.

The IL-1ra beta polypeptides may be in the form of the "mature" protein or may be a part of a larger protein such as a fusion protein. It is often advantageous to include an additional amino acid sequence which contains secretory or leader sequences, pro-sequences, sequences which aid in purification such as multiple histidine residues, or an additional sequence for stability during recombinant production.

Biologically active fragments of the IL-1ra beta polypeptides are also included in the invention. A fragment is a polypeptide having an amino acid sequence that entirely is the same as part, but not all, of the amino acid sequence of the aforementioned IL-1ra beta polypeptides. As with IL-1ra beta polypeptides, fragments may be "free-standing," or comprised within a larger polypeptide of which they form a part or region, most preferably as a single continuous region. Representative examples of polypeptide fragments of the invention, include, for example, fragments from about amino acid number 1-20, 21-40,41-60, 61-80, 81-100, and 101 to the end of IL-1ra beta polypeptide. In this context "about" includes the particularly recited ranges larger or smaller by several, 5, 4, 3, 2 or 1 amino acid at either extreme or at both extremes.

Preferred fragments include, for example, truncation polypeptides having the amino acid sequence of IL-1ra beta polypeptides, except for deletion of a continuous series of residues that includes the amino terminus, or a continuous series of residues that includes the

carboxyl terminus or deletion of two continuous series of residues, one including the amino terminus and one including the carboxyl terminus. Also preferred are fragments characterized by structural or functional attributes such as fragments that comprise alpha-helix and alpha-helix forming regions, beta-sheet and beta-sheet-forming regions, turn and turn-forming regions, coil and coil-forming regions, hydrophilic regions, hydrophobic regions, alpha amphipathic regions, beta amphipathic regions, flexible regions, surface-forming regions, substrate binding region, and high antigenic index regions. Biologically active fragments are those that mediate IL-1ra beta activity, including those with a similar activity or an improved activity, or with a decreased undesirable activity. Also included are those that are antigenic or immunogenic in an animal, especially in a human.

Thus, the polypeptides of the invention include polypeptides having an amino acid sequence at least identical to that of SEQ ID NO:2 or fragments thereof with at least 80% identity to the corresponding fragment of SEQ ID NO:2. Preferably, all of these polypeptides retain the biological activity of the IL-1ra beta, including antigenic activity. Included in this group are variants of the defined sequence and fragments. Preferred variants are those that vary from the referents by conservative amino acid substitutions - i.e., those that substitute a residue with another of like characteristics. Typical such substitutions are among Ala, Val, Leu and Ile; among Ser and Thr; among the acidic residues Asp and Glu; among Asn and Gln; and among the basic residues Lys and Arg; or aromatic residues Phe and Tyr. Particularly preferred are variants in which several, 5-10, 1-5, or 1-2 amino acids are substituted, deleted, or added in any combination.

The IL-1ra beta polypeptides of the invention can be prepared in any suitable manner. Such polypeptides include isolated naturally occurring polypeptides, recombinantly produced polypeptides, synthetically produced polypeptides, or polypeptides produced by a combination of these methods. Means for preparing such polypeptides are well understood in the art.

Polynucleotides of the Invention

Another aspect of the invention relates to isolated polynucleotides which encode the IL-1ra beta polypeptides and polynucleotides closely related thereto.

IL-1ra beta of the invention is structurally related to other proteins of the Interleukin- I family, as shown by the results of sequencing the cDNA encoding human IL-1ra beta. The cDNA sequence contains an open reading frame encoding a protein of 169 amino acids with a deduced molecular weight of 18.7 kDa. IL-1ra beta of FIG. 1 (SEQ ID NO:2) has about 29.9% identity (using BESTFIT (part of GCG suite of programs)) in amino acid residues with human IL-1 receptor antagonist (IL-1ra) (S.P. Eisenberg et al., Nature 343:341-346, 1990) over 162 residues. Furthermore, IL-1ra beta (SEQ ID NO:2) is 21.3% identical to human Interleukin-1 beta (IL-1 beta) over 160 residues (P. E. Auron et al., Proc Natl. Acad. Sci. USA 81:7907-7911, 1984; C. J. March et al., Nature 315:641-647 (1985)). IL-1ra beta gene of FIG. 1 (SEQ ID NO:1) has about 59.0% identity (using BESTFED (part of the GCG suite of programs)) in 230 nucleotide residues with human IL-1ra .

One polynucleotide of the present invention encoding IL-1ra beta may be obtained using standard cloning and screening, from a cDNA library derived from mRNA in cells of human keratinocytes and TNF alpha plus IFN gamma (Interferon gamma) induced epithelial cells using the expressed sequence tag (EST) analysis (Adams, M. D., et al. Science (1991) 252:1651 -1656; Adams, M. D. et al., Nature, (1992) 355:632-634; Adams, M. D., et al., Nature(1995) 377 Supp:3-174). Polynucleotides of the invention can also be obtained from natural sources such as genomic DNA libraries or can be synthesized using well known and commercially available techniques.

Thus, the nucleotide sequence encoding IL-1ra beta polypeptides may be identical over its entire length to the coding sequence in FIG. 1 (SEQ ID NO:1), or may be a degenerate form of this nucleotide sequence encoding the polypeptide of SEQ ID NO:2, or may be highly identical to a nucleotide sequence that encodes the polypeptide of SEQ ID NO:2. Preferably, the polynucleotides of the invention contain a nucleotide sequence that is highly identical, at least identical, with a nucleotide sequence encoding a IL-1ra beta polypeptide, or at least 80% identical with the encoding nucleotide sequence set forth in FIG. 1 (SEQ ID NO:1), or at least 80% identical to a nucleotide sequence encoding the polypeptide of SEQ ID NO:2.

When the polynucleotides of the invention are used for the recombinant production of IL-1ra beta polypeptide, the polynucleotide may include the coding sequence for the mature polypeptide or a fragment thereof, by itself; the coding sequence for the mature polypeptide or fragment in reading frame with other coding sequences, such as those encoding a leader or secretory sequence, a pre-, or pro- or prepro- protein sequence, or other fusion peptide portions. For example, a marker sequence which facilitates purification of the fused polypeptide can be encoded. In certain preferred embodiments of this aspect of the invention, the marker sequence is a hexa-histidine peptide, as provided in the pQE vector (Qiagen, Inc.) and described in Gentz et al., Proc Natl Acad Sci USA (1989) 86:821-824, or is an HA tag. The polynucleotide may also contain non-coding 5' and 3' sequences, such as transcribed, non-translated sequences, splicing and polyadenylation signals, ribosome binding sites and sequences that stabilize mRNA.

Among particularly preferred embodiments of the invention are polynucleotides encoding EL-1ra beta polypeptides having the amino acid sequence of set out in FIG. 1 (SEQ ID NO:2) and variants thereof.

Further preferred embodiments are polynucleotides encoding IL-1ra beta variants that have the amino acid sequence of the IL-1ra beta polypeptide of FIG. 1 (SEQ ID NO:2) in which several, 5-10, 1-5, 1-3, 1-2 or 1 amino acid residues are substituted, deleted or added, in any combination.

Further preferred embodiments of the invention are polynucleotides that are at least 80% identical over their entire length to a polynucleotide encoding the IL-1ra beta polypeptide having the amino acid sequence set out in FIG. 1 (SEQ ID NO:2), and polynucleotides which are complementary to such polynucleotides. In this regard, polynucleotides at least 80% identical over their entire length to the same are particularly preferred, and those with at least 90% are especially preferred. Furthermore, those with at least 97% are highly preferred and those with at least 98-99% are most highly preferred, with at least 99% being the most preferred.

The present invention further relates to polynucleotides that hybridize to the herein above-described sequences. In this regard, the present invention especially relates to polynucleotides which hybridize under stringent conditions to the herein above-described polynucleotides. As herein used, the term "stringent conditions" means hybridization will occur only if there is at least 95% and preferably at least 97% identity between the sequences.

Polynucleotides of the invention, which are sufficiently identical to a nucleotide sequence contained in SEQ ID NO:1, may be used as hybridization probes for cDNA and genomic DNA, to isolate full-length cDNAs and genomic clones encoding IL-1ra beta polypeptide and to isolate cDNA and genomic clones of other genes that have a high sequence similarity to the IL-1ra beta gene. Such hybridization techniques are known to those of skill in the art. Typically these nucleotide sequences are 70% identical, preferably 80% identical, more preferably 90% identical to that of the referent. The probes generally will comprise at least 15 nucleotides. Preferably, such probes will have at least 30 nucleotides and may have at least 50 nucleotides. Particularly preferred probes will range between 30 and 50 nucleotides.

The polynucleotides and polypeptides of the present invention may be employed as research reagents and materials for discovery of treatments and diagnostics to animal and human disease.

Vectors, Host Cells, Expression

The present invention also relates to vectors which comprise a polynucleotide or polynucleotides of the present invention, and host cells which are genetically engineered with vectors of the invention and to the production of polypeptides of the invention by recombinant techniques. Cell-free translation systems can also be employed to produce such proteins using RNAs derived from the DNA constructs of the present invention.

For recombinant production, host cells can be genetically engineered to incorporate expression systems or portions thereof for polynucleotides of the present invention. Introduction of polynucleotides into host cells can be effected by methods described in many standard laboratory manuals, such as Davis et al., BASIC METHODS IN MOLECULAR BIOLOGY (1986) and Sambrook et al., MOLECULAR CLONING: A LABORATORY MANUAL, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989) such as calcium phosphate transfection, DEAE-dextran mediated transfection, transvection, microinjection, cationic lipid-mediated transfection, electroporation, transduction, scrape loading, ballistic introduction or infection.

Representative examples of appropriate hosts include bacterial cells, such as streptococci, staphylococci, E. coli, Streptomyces and Bacillus subtilis cells; fungal cells, such as yeast cells and Aspergillus cells; insect cells such as Drosophila S2 and Spodoptera Sf9 cells; animal cells such as CHO, COS, HeLa, C127, 3T3, BHK, 293 and Bowes melanoma cells; and plant cells.

A great variety of expression systems can be used. Such systems include, among others, chromosomal, episomal and virus-derived systems, e.g., vectors derived from bacterial plasmids, from bacteriophage, from transposons, from yeast episomes, from insertion elements, from yeast chromosomal elements, from viruses such as baculoviruses, papova viruses, such as SV40, vaccinia viruses, adenoviruses, fowl pox viruses, pseudorabies viruses and retroviruses, and vectors derived from combinations thereof, such as those derived from plasmid and bacteriophage genetic elements, such as cosmids and phagemids. The expression systems may contain control regions that regulate as well as engender expression. Generally, any system or vector suitable to maintain, propagate or express polynucleotides to produce a polypeptide in a host may be used. The appropriate nucleotide sequence may be inserted into an expression system by any of a variety of well-known and routine techniques, such as, for example, those set forth in Sambrook et al., MOLECULAR CLONING, A LABORATORY MANUAL (supra).

For secretion of the translated protein into the lumen of the endoplasmic reticulum, into the periplasmic space or into the intracellular environment, appropriate secretion signals may be incorporated into the desired polypeptide. These signals may be endogenous to the polypeptide or they may be heterologous signals.

If the IL-1ra beta polypeptide is to be expressed for use in screening assays, the polypeptide may be produced at the surface of the cell. In this event, the cells may be harvested prior to use in the screening assay. If IL-1ra beta polypeptide is secreted into the medium, the medium can be recovered in order to recover and purify the polypeptide; if produced intracellularly, the cells must first be lysed before the polypeptide is recovered. IL-1ra beta polypeptides can be recovered and purified from recombinant cell cultures by well-known methods including ammonium sulfate or ethanol precipitation, acid extraction, anion or cation

exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxylapatite chromatography and lectin chromatography. Most preferably, high performance liquid chromatography is employed for purification. Well known techniques for refolding proteins may be employed to regenerate active conformation when the polypeptide is denatured during isolation and or purification.

Diagnostic Assays

This invention also relates to the use of IL-1ra beta polynucleotides for use as diagnostic reagents. Detection of a mutated form of IL-1ra beta gene associated with a dysfunction will provide a diagnostic tool that can add to or define a diagnosis of a disease or susceptibility to a disease which results from under-expression, over-expression or altered expression of IL-1ra beta. Individuals carrying mutations in the IL-1ra beta gene may be detected at the DNA level by a variety of techniques.

Nucleic acids for diagnosis may be obtained from a subject's cells, such as from blood, urine, saliva, tissue biopsy or autopsy material. The genomic DNA may be used directly for detection or may be amplified enzymatically by using PCR or other amplification techniques prior to analysis. RNA or cDNA may also be used in similar fashion. Deletions and insertions can be detected by a change in size of the amplified product in comparison to the normal genotype. Point mutations can be identified by hybridizing amplified DNA to labeled IL-1ra beta nucleotide sequences. Perfectly matched sequences can be distinguished from mismatched duplexes by RNase digestion or by differences in melting temperatures. DNA sequence differences may also be detected by alterations in electrophoretic mobility of DNA fragments in gels, with or without denaturing agents, or by direct DNA sequencing. See, e.g., Myers et al., Science (1985) 230:1242. Sequence changes at specific locations may also be revealed by nuclease protection assays, such as RNase and SI protection or the chemical cleavage method. See Cotton et al., Proc Natl Acad Sci USA (1985) 85:4397-4401. In another embodiment, an array of oligonucleotides probes comprising fragments from IL-1ra beta nucleotide sequences can be constructed to conduct efficient screening of e.g., genetic mutations. Array technology methods are well known and have general applicability and can be used to address a variety of questions in molecular genetics including gene expression, genetic linkage, and genetic variability. (See for example: M.Chee et al., Science, Vol 274, pp 610-613 (1996)).

The diagnostic assays offer a process for diagnosing or determining a susceptibility to chronic and acute inflammation, septicemia, arthritis, inflammatory bowel disease, graft vs. host disease, autoimmunity, stroke, cardiac ischemia, acute respiratory disease syndrome (ARDS), psoriasis, restenosis, traumatic brain injury, AIDS, cachexia. through detection of mutation in the IL-1ra beta gene by the methods described.

In addition, chronic and acute inflammation, septicemia, arthritis, inflammatory bowel disease, graft vs. host disease, autoimmunity, stroke, cardiac ischemia, acute respiratory disease syndrome (ARDS), psoriasis, restenosis, traumatic brain injury, AIDS, and cachexia can be diagnosed by methods comprising determining from a sample derived from a subject an abnormally decreased or increased level of IL-1ra beta polypeptide or IL-1ra beta mRNA. Decreased or increased expression can be measured at the RNA level using any of the methods well known in the art for the quantitation of polynucleotides, such as, for example, PCR, RT-PCR, RNase protection, Northern blotting and other hybridization methods. Assay techniques that can be used to determine levels of a protein, such as an IL-1ra beta polypeptide, in a sample derived from a host are well-known to those of skill in the art. Such assay methods include radioimmunoassays, competitive-binding assays, Western Blot analysis and ELISA assays.

Chromosome Assays

The nucleotide sequences of the present invention are also valuable for chromosome identification. The sequence is specifically targeted to and can hybridize with a particular location on an individual human chromosome. The mapping of relevant sequences to chromosomes according to the present invention is an important first step in correlating those sequences with gene associated disease. Once a sequence has been mapped to a precise chromosomal location, the physical position of the sequence on the chromosome can be correlated with genetic map data. Such data are found, for example, in V. McKusick, Mendelian Inheritance in Man (available on line through Johns Hopkins University Welch Medical Library). The relationship between genes and diseases that have been mapped to the same chromosomal region are then identified through linkage analysis (coinheritance of physically adjacent genes).

The IL-1ra beta gene was mapped to chromosome 2, in a region close to IL-1 alpha , beta and IL-1ra by comparing with public databases containing sequences obtained from mapped fragments of genomic DNA obtained by PCR using synthetic oligonucleotide primer pairs.

The differences in the cDNA or genomic sequence between affected and unaffected individuals can also be determined. If a mutation is observed in some or all of the affected individuals but not in any normal individuals, then the mutation is likely to be the causative agent of the disease.

Antibodies

The polypeptides of the invention or their fragments or analogs thereof, or cells expressing them can also be used as immunogens to produce antibodies immunospecific for the IL-1ra beta polypeptides. The term"immunospecific" means that the antibodies have substantiall greater affinity for the polypeptides of the invention than their affinity for other related polypeptides in the prior art.

Antibodies generated against the IL-1ra beta polypeptides can be obtained by administering the polypeptides or epitope-bearing fragments, analogs or cells to an animal, preferably a nonhuman, using routine protocols. For preparation of monoclonal antibodies, any technique which provides antibodies produced by continuous cell line cultures can be used. Examples include the hybridoma technique (Kohler, G. and Milstein, C., Nature (1975) 256:495497), the trioma technique, the human B-cell hybridoma technique (Kozbor et al., Immunology Today (1983) 4:72) and the EBV-hybridoma technique (Cole et al., MONOCLONAL ANTIBODIES AND CANCER THERAPY, pp. 77-96, Alan R. Liss, Inc., 1985).

Techniques for the production of single chain antibodies (U.S. Pat. No. 4,946,778) can also be adapted to produce single chain antibodies to polypeptides of this invention. Also, transgenic mice, or other organisms including other mammals, may be used to express humanized antibodies.

The above-described antibodies may be employed to isolate or to identify clones expressing the polypeptide or to purify the polypeptides by affinity chromatography.

Antibodies against IL-1ra beta polypeptides may also be employed to treat chronic and acute inflammation, septicemia, arthritis, inflammatory bowel disease, graft vs. host disease, autoimmunity, stroke, cardiac ischemia, acute respiratory disease syndrome (ARDS), psoriasis, restenosis, traumatic brain injury, AIDS, cachexia., among others.

Vaccines

Another aspect of the invention relates to a method for inducing an immunological response in a mammal which comprises inoculating the mammal with IL-1ra beta polypeptide, or a fragment thereof, adequate to produce antibody and/or T cell immune response to protect said animal from chronic and acute inflammation, septicemia, arthritis, inflammatory bowel disease, graft vs. host disease, autoimmunity, stroke, cardiac ischemia, acute respiratory disease syndrome (ARDS), psoriasis, restenosis, traumatic brain injury, AIDS, cachexia., among others. Yet another aspect of the invention relates to a method of inducing immunological response in a mammal which comprises, delivering IL-1ra beta gene via a vector directing expression of IL-1ra beta polypeptide in vivo in order to induce such an immunological response to produce antibody to protect said animal from diseases.

Further aspect of the invention relates to an immunological/vaccine formulation (composition) which, when introduced into a mammalian host, induces an immunological response in that mammal to a IL-1ra beta polypeptide wherein the composition comprises a IL-1ra beta polypeptide or IL-1ra beta gene. The vaccine formulation may further comprise a suitable carrier. Since IL-1ra beta polypeptide may be broken down in the stomach, it is preferably administered parenterally (including subcutaneous, intramuscular, intravenous, intradermal etc. injection). Formulations suitable for parenteral administration include aqueous and non-aqueous sterile injection solutions which may contain anti-oxidants, buffers, bacteriostats and solutes which render the formulation instonic with the blood of the recipient; and aqueous and non-aqueous sterile suspensions which may include suspending agents or thickening agents. The formulations may be presented in unit-dose or multi-dose containers, for example, sealed ampoules and vials and may be stored in a freeze-dried condition requiring only the addition of the sterile liquid carrier immediately prior to use. The vaccine formulation may also include adjuvant systems for enhancing the immunogenicity of the formulation, such as oil-in water systems and other systems known in the art. The dosage will depend on the specific activity of the vaccine and can be readily determined by routine experimentation.

Screening Assays

The IL-1ra beta polypeptide of the present invention may be employed in a screening process for compounds which stimulate (agonists) or inhibit (antagonists, or otherwise called inhibitors) the synthesis or action of the IL-1ra beta polypeptide of the present invention. The IL-1ra beta polypeptide of the present invention may also be employed in a screening process for compounds which mimic the agonist or antagonist properties of the IL-1ra beta polypeptide of the present invention. Thus, polypeptides of the invention may also be used to assess and identify agonist or antagonists from, for example, cells, cell-free preparations, chemical libraries, and natural product mixtures. These agonists or antagonists may be natural substrates, ligands, receptors, etc., as the case may be, of the polypeptide of the present invention; or may be structural or functional mimetics of the polypeptide of the present invention. See Coligan et al., Current Protocols in Immunology 1(2):Chapter 5 (1991).

IL-1ra beta proteins are ubiquitous in the mammalian host and are responsible for many biological functions, including many pathologies. Accordingly, it is desirous to find compounds and drugs which stimulate IL-1ra beta polypeptide on the one hand and which can inhibit the function of IL-1ra beta polypeptide on the other hand. In general, agonists are employed for therapeutic and prophylactic purposes for such conditions as chronic and acute inflammation, septicemia, arthritis, inflammatory bowel disease, graft vs. host disease, autoimmunity, stroke, cardiac ischemia, acute respiratory disease syndrome (ARDS), psoriasis, restenosis, traumatic brain injury, AIDS, cachexia. Antagonists may be employed for a variety of therapeutic and prophylactic purposes for such conditions as chronic and acute inflammation, septicemia, arthritis, inflammatory bowel disease, graft vs. host disease, autoimmunity,

stroke, shock, atherschlerosis, cardiac ischemia, acute respiratory disease syndrome (ARDS), psoriasis, restenosis, traumatic brain injury, AIDS, cachexia.

In general, such screening procedures may involve identifying, generating and using appropriate cells which express the receptor of the IL-1ra beta polypeptide of the present invention on the surface thereof. Such cells include cells from mammals, yeast, Drosophila or E. coli. Such cells may be identified, for example, by direct binding methods using radiolabeled or fluorescently tagged IL-1ra beta polypeptide. Cells expressing the IL-1ra beta polypeptide receptor (or cell membrane containing the expressed polypeptide) are then contacted with a test compound to observe binding, or stimulation or inhibition of a functional response. Alternatively, the cDNA for the IL-1ra beta polypeptide receptor may be cloned by the above direct binding methods using expression cloning or purification methods known in the art, and its intracellular domain expressed as a secreted or membrane protein. The soluble or membrane bound receptor can then be used to identify agonists or antagonists via direct binding methods.

The assays may simply test binding of a candidate compound wherein adherence to the cells bearing the IL-1ra beta polypeptide receptor is detected by means of a label directly or indirectly associated with the candidate compound or in an assay involving competition with a labeled IL-1ra beta polypeptide. Further, these assays may test whether the candidate compound results in a signal similar to that generated by binding of the IL-1ra beta polypeptide, using detection systems appropriate to the cells bearing the IL-1ra beta polypeptide receptor at their surfaces. Inhibitors of activation are generally assayed in the presence of a known agonist and the effect on activation by the agonist by the presence of the candidate compound is observed. Standard methods for conducting such screening assays are well understood in the art.

Examples of potential IL-1ra beta polypeptide antagonists include antibodies or, in some cases, oligonucleotides or proteins which are closely related to the ligands, substrates, receptors, etc., as the case may be, of the IL-1ra beta polypeptide, e.g., a fragment of the ligands, substrates, receptors, or small molecules which bind to the target receptor of the present invention but do not elicit a response, so that the activity of the polypeptide is prevented.

Prophylactic and Therapeutic Methods

This invention provides methods of treating an abnormal conditions related to both an excess of and insufficient amounts of IL-1ra beta polypeptide activity.

If the activity of IL-1ra beta polypeptide is in excess, several approaches are available. One approach comprises administering to a subject an inhibitor compound (antagonist) as herein above described along with a pharmaceutically acceptable carrier in an amount effective to inhibit activation by blocking binding of the IL-1ra beta polypeptide to its target receptor, or by inhibiting a second signal, and thereby alleviating the abnormal condition.

In another approach, soluble forms of EL-1ra beta polypeptides capable of binding its receptor in competition with endogenous IL-1ra beta polypeptide may be administered. Typical embodiments of such competitors comprise fragments of the IL-1ra beta polypeptide.

In still another approach, expression of the gene encoding endogenous IL-1ra beta polypeptide can be inhibited using expression blocking techniques. Known such techniques involve the use of antisense sequences, either internally generated or separately administered. See, for example, O'Connor, J Neurochem (1991) 56:560 in Oligodeoxynucleotides as Antisense Inhibitors of Gene Expression, CRC Press, Boca Raton,

Fla. (1988). Alternatively, oligonucleotides which form triple helices with the gene can be supplied. See, for example, Lee et al., Nucleic Acids Res (1979) 6:3073; Cooney et al, Science (1988) 241:456; Dervan et al, Science (1991) 251:1360. These oligomers can be administered per se or the relevant oligomers can be expressed in vivo.

For treating abnormal conditions related to an under-expression of IL-1ra beta and its activity, several approaches are also available. One approach comprises administering to a subject a therapeutically effective amount of the IL-1ra beta polypeptide or a compound, i.e., an agonist or mimetic as described above, in combination with a pharmaceutically acceptable carrier, to thereby alleviate the abnormal condition. Alternatively, gene therapy may be employed to effect the endogenous production of IL-1ra beta by the relevant cells in the subject. For example, a polynucleotide of the invention may be engineered for expression in a replication defective retroviral vector, as discussed above. The retroviral expression construct may then be isolated and introduced into a packaging cell transduced with a retroviral plasmid vector containing RNA encoding a polypeptide of the present invention such that the packaging cell now produces infectious viral particles containing the gene of interest. These producer cells may be administered to a subject for engineering cells in vivo and expression of the polypeptide in vivo. For overview of gene therapy, see Chapter 20, Gene Therapy and other Molecular Genetic-based Therapeutic Approaches, (and references cited therein) in Human Molecular Genetics, T Strachan and A P Read, BIOS Scientific Publishers Ltd (1996).

Formulation and Administration

Peptides, such as the soluble form of IL-1ra beta polypeptides, and agonists and antagonist peptides or small molecules, may be formulated in combination with a suitable pharmaceutical carrier. Such formulations comprise a therapeutically effective amount of the polypeptide or compound, and a pharmaceutically acceptable carrier or excipient. Such carriers include but are not limited to, saline, buffered saline, dextrose, water, glycerol, ethanol, and combinations thereof. Formulation should suit the mode of administration, and is well within the skill of the art. The invention further relates to pharmaceutical packs and kits comprising one or more containers filled with one or more of the ingredients of the aforementioned compositions of the invention.

Polypeptides and other compounds of the present invention may be employed alone or in conjunction with other compounds, such as therapeutic compounds.

Preferred forms of systemic administration of the pharmaceutical compositions include injection, typically by intravenous injection. Other injection routes, such as subcutaneous, intramuscular, or intraperitoneal, can be used. Alternative means for systemic administration include transmucosal and transdermal administration using penetrants such as bile salts or fusidic acids or other detergents. In addition, if properly formulated in enteric or encapsulated formulations, oral administration may also be possible. Administration of these compounds may also be topical and/or localized, in the form of salves, pastes, gels and the like.

The dosage range required depends on the choice of peptide, the route of administration, the nature of the formulation, the nature of the subject's condition, and the judgment of the attending practitioner. Suitable dosages, however, are in the range of 0.1-100 mu g/kg of subject. Wide variations in the needed dosage, however, are to be expected in view of the variety of compounds available and the differing efficiencies of various routes of administration. For example, oral administration would be expected to require higher dosages than administration by intravenous injection. Variations in these dosage levels can be adjusted using standard empirical routines for optimization, as is well understood in the art.

Polypeptides used in treatment can also be generated endogenously in the subject, in treatment modalities often referred to as "gene therapy" as described above. Thus, for example, cells from a subject may be engineered with a polynucleotide, such as a DNA or RNA, to encode a polypeptide ex vivo, and for example, by the use of a retroviral plasmid vector. The cells are then introduced into the subject.

EXAMPLES

The examples below are carried out using standard techniques, which are well known and routine to those of skill in the art, except where otherwise described in detail. The examples illustrate, but do not limit the invention.

Example 1

Isolation and identification of IL-1RA beta

A potential full length clone (HGS EST #1506331; Project ID HAICQ62) was initially identified through a search of the Human Genome Sciences EST database (vide supra for EST analysis) for proteins with homology to members of the interleukin 1 family. This partial sequence showed significant seqeunce identity (35% over 77 aa) to murine IL-1ra. This cDNA was completely sequenced on both strands using an automated sequencer. A total of 1183 bp were sequenced, and this includes an open reading frame enconding a peptide of 169 aa. The cDNA and protein sequences are SEQ ID NOS: 1 and 2, respectively, and are named IL-1 ra beta . The protein does not appear to have a signal sequence at its amino terminus and is likely to be expressed as an intracellular, cytosolic protein like other members of the family. It is possible that alternative splice forms exist which include a signal sequence, as has been found for IL-1ra.

The IL-1ra beta gene was mapped to chromosome 2, in a region close to IL-1 alpha , and beta and IL-1ra by comparing with public databases containing sequences obtained from mapped fragments of genomic DNA obtained by PCR using synthetic oligonucleotide primer pairs. Using the algorithm BLAST, a match was found with human STS CHLC.GAAT11C03.P3330 clone GAAT11IC03 (Accession number G942011) which can be mapped to chromosome 2 approximately 142 cM from the top of the chromosome.

SEQUENCE LISTING
- - (1) GENERAL INFORMATION:
- (iii) NUMBER OF SEQUENCES: 2
- - (2) INFORMATION FOR SEQ ID NO:1:
- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1183 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: cDNA
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:
- GGCACGAGCCACGATTCAGTCCCCTGGACTGTAGATAAAGACCCTTTCTTGCCAGGTGCT60
- GAGACAACCACACTATGAGAGGCACTCCAGGAGACGCTGATGGTGGAGGAAGGGCCGTCT120
- ATCAATCAATGTGTAAACCTATTACTGGGACTATTAATGATTTGAATCAGCAAGTGTGGA180
- CCCTTCAGGGTCAGAACCTTGTGGCAGTTCCACGAAGTGACAGTGTGACCCCAGTCACTG240

- TTGCTGTTATCACATGCAAGTATCCAGAGGCTCTTGAGCAAGGCAGAGGGGATCCCATTT300
- ATTTGGGAATCCAGAATCCAGAAATGTGTTTGTATTGTGAGAAGGTTGGAGAACAGCCCA360
- CATTGCAGCTAAAAGAGCAGAAGATCATGGATCTGTATGGCCAACCCGAGCCCGTGAAAC420
- CCTTCCTTTTCTACCGTGCCAAGACTGGTAGGACCTCCACCCTTGAGTCTGTGGCCTTCC480
- CGGACTGGTTCATTGCCTCCTCCAAGAGAGACCAGCCCATCATTCTGACTTCAGAACTTG540
- GGAAGTCATACAACACTGCCTTTGAATTAAATATAAATGACTGAACTCAGCCTAGAGGTG600
- GCAGCTTGGTCTTTGTCTTAAAGTTTCTGGTTCCCAATGTGTTTTCGTCTACATTTTCTT660
- AGTGTCATTTTCACGCTGGTGCTGAGACAGGGGCAAGGCTGCTGTTATCATCTCATTTTA720
- TAATGAAGAAGAAGCAATTACTTCATAGCAACTGAAGAACAGGATGTGGCCTCAGAAGCA780
- GGAGAGCTGGGTGGTATAAGGCTGTCCTCTCAAGCTGGTGCTGTGTAGGCCACAAGGCAT840
- CTGCATGAGTGACTTTAAGACTCAAAGACCAAACACTGAGCTTTCTTCTAGGGGTGGGTA900
- TGAAGATGCTTCAGAGCTCATGCGCGTTACCCACGATGGCATGACTAGCACAGAGCTGAT960
- CTCTGTTTCTGTTTTGCTTTATTCCCTCTTGGGATGATATCATCCAGTCTTTATATGTTG1020
- CCAATATACCTCATTGTGTGTAATAGAACCTTCTTAGCATTAAGACCTTGTAAACAAAAA1080
- TAATTCTTGTGTTAAGTTAAATCATTTTTGTCCTAATTGTAATGTGTAATCTTAAAGTTA1140
- AATAAACTTTGTGTATTTATATAATAAAAAAAAAAAAAAAAAAA1183
- - (2) INFORMATION FOR SEQ ID NO:2:
- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 169 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: protein
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:
- MetArgGlyThrProGlyAspAlaAspGlyGlyGlyArgAlaValTyr
151015
- GlnSerMetCysLysProIleThrGlyThrIleAsnAspLeuAsnGln
202530
- GlnValTrpThrLeuGlnGlyGlnAsnLeuValAlaValProArgSer
354045
- AspSerValThrProValThrValAlaValIleThrCysLysTyrPro
505560
- GluAlaLeuGluGlnGlyArgGlyAspProIleTyrLeuGlyIleGln
65707580
- AsnProGluMetCysLeuTyrCysGluLysValGlyGluGlnProThr
859095
- LeuGlnLeuLysGluGlnLysIleMetAspLeuTyrGlyGlnProGlu
100105110
- ProValLysProPheLeuPheTyrArgAlaLysThrGlyArgThrSer
115120125
- ThrLeuGluSerValAlaPheProAspTrpPheIleAlaSerSerLys
130135140
- ArgAspGlnProIleIleLeuThrSerGluLeuGlyLysSerTyrAsn
145150155160
- ThrAlaPheGluLeuAsnIleAsnAsp
165

CLAIMS: What is claimed is:

[*1] 1. An isolated polynucleotide comprising a polyncleotide which encodes the IL-1ra beta polypeptide of SEQ ID NO:2.

[*2] 2. An isolated polynucleotide comprising a polynucleotide which is at least 80% identical to that of SEQ ID NO:1 in which identity is calculated using FASTA, and parameters are set so that the highest order match is obtained.

[*3] 3. An isolated polynucleotide comprising a polynucleotide contained in SEQ ID NO:1 which encodes the IL-1ra beta polypeptide of SEQ ID NO:2.

[*4] 4. The polynucleotide of SEQ ID NO: 1.

[*5] 5. An isolated polynucleotide of claim 3 comprising a polynucleotide that has at least 90% identy to that of SEQ ID NO:1, in which identity is calculated using FASTA, and parameters are set so that the highest order match is obtained.

[*6] 6. An isolated polynucleotide of claim 2 comprising a polynucleotide that has at least 95% identity to that of SEQ ID NO:1, in which identity is calculated using FASTA, and parameters are set so that the highest order match is obtained.

[*7] 7. An isolated polynucleotide comprising a polynucleotide that has at least 80% identity to the sequence contained in SEQ ID NO:1 encoding the polypeptide of SEQ ID NO:2, in which identity is calculated using FASTA, and parameters are set so that the highest order match is obtained.

[*8] 8. An isolated polynucleotide of claim 7 comprising a polynucleotide that has at least 90% identity to the sequence contained in SEQ ID NO:1 encoding a polypeptide of SEQ ID NO:2, in which identity is calculated using FASTA, and parameters are set so that the highest order match is obtained.

[*9] 9. An isolated polynucleotide comprising a polynucleotide that has at least 95% identity to the sequence contained in SEQ ID NO:1 encoding a polypeptide of SEQ ID NO:2, in which identity is calculated using FASTA, and parameters are set so that the highest order match is obtained.

[*10] 10. An isolated polynucleotide which is complementary to a polynucleotide of any one of claims 1, 3, 7, 8, and 9.

[*11] 11. The polynucleotide which is the RNA transcript of SEQ ID NO:1.

[*12] 12. The isolated polynucleotide which is the RNA transcript of coding region nucleotide number 75 to 581 of SEQ ID NO:1.

[*13] 13. A polynucleotide comprising a DNA sequence obtainable by screening an appropriate library under stringent hybridization conditions with a probe having the sequence of SEQ ID NO:1 ;and isolating said DNA sequence.

[*14] 14. The polynucleotide of claim 1 which is DNA or RNA.

[*15] 15. A DNA or RNA molecule comprising an expression system, wherein said expression system is capable of production a IL-1ra beta polypeptide of SEQ ID NO:2 when

said expression system is present in a compatible host cell.

[*16] 16. A host cell comprising the expression system of claim 15.

[*17] 17. A process for producing a IL-1ra beta polypeptide comprising culturing a host of claim 16 under conditions sufficient for the production of said polypeptide, and recovering the polypeptide from the culture.

[*18] 18. A process for producing a cell which produces a IL-1ra beta polypeptide thereof comprising transforming or transfecting a host cell with the expression system of claim 15, such that the host cell, under appropriate culture conditions, produces a IL-1ra beta polypeptide.

[*19] 19. Cells produced by the process of claim 18.

Pat. No. 5,075,222, *

5,075,222

♦ GET 1st DRAWING SHEET OF 22

Dec. 24, 1991

Interleukin-1 inhibitors

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ASSIGNEE-AFTER-ISSUE: Date Transaction Recorded: Apr. 09, 1992
FREE FORM TEXT
JOINT INTEREST TO ESTABLISH JOINT OWNERSHIP BY SYNERGEN, INC . AND THE
UNIVERSITY OF COLORADO FOUNDATION, INC. (SEE DOCUM ENT FOR DETAILS).
UNIVERSITY OF COLORADO FOUNDATION, INC. A CORPORATION OF CO 1305 UNIVERSITY
AVENUE BOULDER, COLORADO 80301
Reel & Frame Number: 006073/0346

Date Transaction Recorded: Mar. 26, 1999
CHANGE OF NAME (SEE DOCUMENT FOR DETAILS).
AMGEN BOULDER INC., C/O AMGEN INC. ONE AMGEN CENTER THOUSAND OAKS,
CALIFORNIA 91320
Reel & Frame Number: 009845/0143

Date Transaction Recorded: Mar. 26, 1999
MERGER (SEE DOCUMENT FOR DETAILS).
AMGEN INC. ONE AMGEN CENTER THOUSAND OAKS, CALIFORNIA 91320
Reel & Frame Number: 009845/0147

APPL-NO: 506,522

FILED: Apr. 6, 1990

CERTCORR: Mar. 07, 1995 a Certificate of Correction was issued for this Patent

REL-US-DATA:
Continuation of Ser. No. 266,531, Nov. 3, 1988 now abandoned Which is a continuation-in-part of Ser. No. 248,521, Sep. 23, 1988 now abandoned Which is a continuation-in-part of Ser. No. 238,713, Aug. 31, 1988 now abandoned Which is a continuation-in-part of Ser. No. 199,915, May 27, 1988 now abandoned

INT-CL: [5] C12P 21#02; C12P 19#34; C12N 15#00; C12N 7#00; C12N 5#00; C12N 1#21; C12N 15#70; C12N 15#79; C12N 15#81; C12N 1#16; C07H 15#12; C07K 3#00

US-CL: 435#69.1; 435#91.41; 435#235.1; 435#252.3; 435#252.33; 435#320.1;

435#360; 435#455; 435#461; 435#485; 435#488; 530#350; 536#23.51;

CL: 435;530;536;

SEARCH-FLD: 435#69.1, 91, 172.3, 253.33, 320, 235; 536#27; 530#350; 935#4, 18, 29, 32, 34, 38, 56, 57, 62, 70, 72, 77, 82, 320.1, 240.1, 255

REF-CITED:

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		World Intellectual Property
WO89/01946	3/1989	* Organization

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CORE TERMS: sequence, cell, inhibitor, protein, vector, host, gene, isolated, purified, cdna, coli, fraction, amino acid, species, monocyte, coding, promoter, plasmid, leader, encode, alpha, operational, column, beta, gel, microorganism, supernatant, residue, medium, marker

ABST:
DNA sequences that encode Interleukin-1 inhibitors and recombinant-DNA methods for the production of interleukin-1 inhibitors are provided. The DNA sequences encode proteins having interleukin-1 inhibitors activity.

NO-OF-CLAIMS: 31

EXMPL-CLAIM: 17

NO-OF-FIGURES: 26

NO-DRWNG-PP: 22

PARCASE:

This application is a continuation of application Ser. No. 07/266,531, filed Nov. 3, 1988, now abandoned, which is a continuation-in-part of Ser. No. 07/248,521, filed Sept. 23, 1988, now abandoned, which is a continuation-in-part of Ser. No. 07/238,713, filed Aug. 31, 1988, now abandoned, which is a continuation-in-part of Ser. No. 07/199,915, filed May 27, 1988, now abandoned.
A. IL-1

Interleukins-1 are a class of proteins produced by numerous cell-types, including monocytes and some macrophages. This class includes at least two 17-18 kilodalton proteins known as interleukin-1 alpha and interleukin-1 beta. These proteins have important physiological effects on a number of different target cells involved in the inflammatory and immune responses. The proteins are co-mitogens (with phytohemaglutinin) for T-cells, cause both fibroblasts and chondrocytes to secrete latent collagenase, and increase the surface adhesive powers of endothelial cells for neutrophils. In addition, they act on the hypothalamus as pyrogens, they stimulate the catabolism of muscle protein, and they cause hepatocytes to synthesize a class of proteins known as "acute phase reactants." Thus, interleukins-1 (IL-1) are obviously an important part of an organism's response to infection and injury.
B. Pathological Roles of IL-1

However, despite their normally teneficial effects, circumstances have come to light in which the actions of IL-1 are harmful. For example, IL-1 may increase the level of collagenase in an arthritic joint and has been implicated as a mediator of both the acute and chronic stages of immunopathology in rheumatoid arthritis. IL-1 may be responsible for altering endothelial cell function, directing the chemotaxis and migration of leukocytes and lymphocytes into the synovial tissue, inducing capillary proliferation and stimulating macrophage accumulation in the synovial lining during the acute phase of this disease. In the phase of tissue destruction, IL-1 has been implicated as a mediator in induction of tissue damage through stimulating release of enzymes from fibroblasts and chondrocytes.

In addition, excessive IL-1 production has been demonstrated in the skin of patients with psoriasis and high levels of IL-1 can be found in the synovial fluid of patients with psoriatic arthritis. IL-1 released by cells in the inflamed synovium in psoriatic arthritis may mediate tissue destruction through stimulation of enzyme release from other cells. The joint pathology of Reiter's syndrome is similar to that seen in psoriatic arthritis and in rheumatoid arthritis. IL-1 has been implicated as a mediator of tissue destruction in these three different forms of inflammatory arthritis. Moreover, IL-1 may be found in the synovial fluid of patients with osteoarthritis. The release of IL-1 by chondrocytes has been implicated in the destruction of articular cartilage in this disease.

IL-1 may also increase the severity of autoimmune diseases. For example, decreased IL-1 production has been described from peripheral blood cells in persons suffering from systemic lupus erythematosus. Moreover, some of the alterations in B lymphocyte function may be related to abnormalities in IL-1 production or IL-1 availability.

Excessive IL-1 production has been demonstrated in the peripheral monocytes of patients with scleroderma, and IL-1 has been implicated as a possible agent of fibrosis through stimulation of collagen production by fibroblasts. The mechanism of tissue damage in dermatomyositis might also involve cell-mediated immunity and IL-1 may therefore be involved as a mediator in this pathophysiological process.

Acute and chronic interstitial lung disease is characterized by excessive collagen production by lung fibroblasts which may be stimulated by IL-1. Recent studies on an animal model of pulmonary hypertension indicate that IL-1 may be responsible for induction of endothelial cell changes that result in narrowing of pulmonary arteries It is this narrowing that leads to pulmonary hypertension and further secondary damage. Thus, IL-1 inhibitors could be useful in treating these lung diseases.

Recent studies have described that IL-1 is capable of directly damaging the beta cells in the Islets of Langerhans that are responsible for the production of insulin. IL-1 damage to the cells is now hypothesized to be a primary event in the acute phase of juvenile diabetes mellitus.

Monocyte and macrophage infiltration in the kidneys predominates in many forms of acute and chronic glomerulonephritis. IL-1 release by these cells may result in local accumulation of other inflammatory cells, eventually leading to inflammatory damage and fibrotic reaction in the kidneys.

It has been demonstrated that the crystals found in tissues or fluids in gout or pseudogout can directly stimulate macrophages to release IL-1. Thus, IL-1 may be an important mediator in the inflammatory cycle in these diseases.

IL-1 is capable of inducing loss of calcium from bones and may be responsible for the osteoporosis that is seen in inflammatory joint diseases.

Keratinocytes from patients with psoriasis release large amounts of IL-1. This mediator may be responsible for the secondary cell proliferation and accumulation which occurs in the skin in patients with this disease.

IL-1 is one of the important endogenous pyrogens and may be responsible for inducing the marked degree of fever seen in some infectious diseases such as acute febrile illnesses due to bacteria or viruses.

Sarcoidosis is characterized by granulomatous lesions in many different organs in the body. IL-1 has been shown to be capable of inducing granuloma formation in vitro and may be involved in this process in patients with sarcoidosis.

Excessive IL-1 production has been demonstrated in peripheral monocytes from both Crohn's disease and ulcerative colitis. Local IL-1 release in the intestine may be an important mediator in stimulating the inflammatory cycle in these diseases.

Certain lymphomas are characterized by fever, osteoporosis and even secondary arthritis. Excessive IL-1 release has been demonstrated by some lymphoma cells in vitro and may be responsible for some of the clinical manifestations of these malignancies. Also, IL-1 production by some malignant lymphocytes may be responsible for some of the fever, acute phase response and cachexia seen with leukemias.

IL-1 release by astrocytes in the brain is thought to be responsible for inducing the fibrosis that may result after damage to the brain from vascular occlusion.

C. Uses for an IL-1 Inhibitor

In these and other circumstances in which IL-1 has a harmful effect, there is clearly a clinical use for an inhibitor of IL-1 action. As IL-1 is a co-mitogen for T-cells, it is central to the development of autoimmune and other immune diseases. Thus, systemically administered, IL-1 inhibitors could be useful immunosuppressive agents. Locally applied, such IL-1 inhibitors could serve to prevent tissue destruction in an inflamed joint and other sites of

inflammation. Indeed, to prevent tissue destruction some IL-1 inhibitors could be even more effective when administered in conjunction with collagenase inhibitors.

Therapeutic intervention against the action of IL-1 might be possible at the level of synthesis, secretion, or the target cell's binding or response to the protein. IL-1 is synthesized by monocyte/macrophages and other cells in response to lipopolysaccharides, complement fragments and viruses. Any molecule that blocks binding of these inducing agents to producer cells or which interferes with their effects on the physiology of these cells would serve as a regulator of IL-1 action. IL-1 is not secreted by a traditional secretion system since mRNAs have been isolated that code for at least two 30 kd precursors of the proteins but which do not contain a hydrophobic signal sequence. Release of the active protein from the inactive precursor probably requires proteolysis of that precursor. An inhibitor of the release of IL-1 or IL-Is from their precursors could theoretically regulate IL-1 action. IL-1 probably acts on target cells through a classical receptor-mediated pathway, although that receptor has not yet been isolated. Thus, it could be that a molecule that interferes with IL-1 binding to its receptors, or down-regulates these receptors, could also regulate IL-1 action. Moreover, although the intracellular events following receptor binding of IL-1 are not yet fully understood, it is possible that agents exist that can interfere with the cellular responses to other receptor-mediated events and therefore block IL-1 action. For the reasons stated above, proteins and small molecules capable of inhibiting IL-1 in one or more of these manners have been sought.

Surprisingly, the present inventors have found at least two IL-1 inhibitor proteins with IL-1 inhibiting properties. These molecules have been obtained in a purified form which will enable one of ordinary skill in the art to determine their amino acid sequence. Furthermore, a preparation of cells has been characterized which produce these proteins, and an mRNA that leads to its synthesis has been characterized. Finally, an antisera has been developed that will facilitate screening of cDNA expression libraries for the genes coding for these inhibitors. Together these reagents will allow cDNAs encoding the IL-1 inhibitors to be cloned. These genes will, in turn, make possible the large scale production of IL-1 inhibitors suitable for use in pharmaceutical formulations useful in treating pathophysicological conditions mediated by IL-1.

SUMMARY OF THE INVENTION

This invention relates to IL-1 inhibitors ("IL-li") generally and, more specifically, to a monocyte-derived IL-1 inhibitor. Additionally, the present invention relates to biologically-active analogs of these inhibitors.

An object of the present invention is to provide purified forms of IL-1 inhibitors which are active against IL-1 alpha or IL-1 beta or a combination thereof. An additional object of the present invention is to provide these inhibitors in purified forms to enable the determination of their amino acid sequence. A further object is to provide the amino acid sequences of certain IL-1 inhibitors. Furthermore, the identification of biologically-active analogs of such IL-1 inhibitors with enhanced or equivalent properties is also one of the objects of the invention.

Additionally, it is an object of this invention to provide a recombinant-DNA system for the production of the IL-1 inhibitors described herein. A further object cf the present invention includes providing purified forms of IL-1 inhibitors which would be valuable as pharmaceutical preparations exhibiting activity against IL-1.

Additional objects and advantages of the invention will be set forth in part in the description which follows, and in part will be obvious from the description or may be learned from the practice of the invention. The objects and advantages may be realized and attained by means of the instrumentalities and combinations particularly pointed out in the appended claims.

To achieve the objects and in accordance with the purposes of the present invention, IL-1 inhibitors are disclosed which exhibit inhibitory activity against IL-1. The preferred inhibitors have been isolated in a purified form from monocyte-conditioned medium with monocytes grown on IgG-coated plates.

Preferred inhibitors of the present invention are 1, 2 and 3. Inhibitors 1 and 2 are proteins running at positions characteristic of 22-23 kDa proteins on SDS-PAGE and eluting at 52 mM and 60 mM NaCl, respectively, from a Mono Q FPLC column under specified conditions. Inhibitor 3 is a protein running at a position characteristic of a 20 kD protein on SDS-PAGE and eluting at 48 mM NaCl from a Mono Q FPLC column under the specified conditions. Additionally, to achieve the objects and in accordance with the purposes of the present invention, pharmaceutical compositions containing, at least one of the active ingredients, an IL-1 inhibitor in accordance with the present invention or its biologically-active analog as set forth herein are disclosed.

Moreover, to achieve the objects and in accordance with the purposes of the present invention, a recombinant-DNA system for the creation of these IL-1 inhibitors and their analogs is also disclosed. A preferred embodiment of this system includes at least one cDNA clone or its synthetic equivalent encoding at least one IL-1 inhibitor along with vectors and cells constituting an expression system capable of expressing the IL-1 inhibitors disclosed herein. Antisera for use in identifying these cDNA clones is also provided. Expression systems for producing these IL-1 inhibitors using these cDNA clones, their analogs, or other DNA sequences encoding these inhibitors are also provided.

DRWDESC:
BRIEF DESCRIPTION OF THE FIGURES

FIGS. 1a and 1b depict the protein profile of the Mono Q chromatography of two metabolically-labelled monocyte supernatants. The cells were cultured on IgG (1a) or fetal calf serum (1b) coated plates.

FIG. 2a shows silver stained gels of fractions from the regions indicated in FIGS. 1a and 1b.

FIG. 2b is an autoradiogram of the gels shown in FIG. 2a.

FIGS. 3a, b and c present data on the purified IL-li of Example 1. FIG. 3a presents chromatography data with the radioactivity pattern superimposed. FIG. 3b presents silver stained gels run on samples of the fractions indicated in FIG. 3a. FIG. 3c presents autoradiograms of the gels in FIG. 3b.

FIGS. 4a and b present the results of gel filtration chromatograms of Mono Q-purified IL-li.

FIGS. 5a and b present Western analysis of mouse antisera.

FIG. 6 depicts the construction of plasmid pSVXVPL21L-li.

FIG. 7 depicts the construction of plasmid pMK-SGE:IL-li.

FIGS. 8a-d present data on IL-li- alpha . FIGS. 8a and 8b present chromotography data. FIG. 8c presents a silver stained gel run on samples of fractions indicated in FIG. 8b. FIG. 8d presents an autoradiogram.

FIGS. 9a and 9b present data on IL-li- beta . FIG. 9a presents chromotography data. FIG. 9b presents SDS-PAGE data.

FIG. 10 presents data of IL-li- alpha peptide separation.

FIG. 11 presents data of IL-li- beta peptide separation.

FIG. 12A is a photograph of the gel with the GT10-ILli-2A digested with EcoRI after electrophoresis according to Example 6.

FIG. 12B presents data of an autoradiogram of a Southern blot of the gel shown in FIG. 12A.

FIG. 13 depicts a part of the DNA sequence of the protein coding region of lambda GT10-ILli-2A and the predicted amino acid sequence according to Example 6.

FIG. 14 depicts the nucleotide sequence of GT10-illI-2A.

FIG. 15 depicts a peptide including, inter alia, an IL-li sequence and a secretory leader sequence.

DETDESC:

DESCRIPTION OF THE PREFERRED EMBODIMENT

Reference will now be made in detail to the presently preferred embodiments of the invention, which, together with the following examples, serve to explain the principles of the invention.
A. Inhibitor from Human Monocytes

As noted above, the present invention relates to IL-1 inhibitors which have been isolated in a purified form. Preferably, the IL-1 inhibitors of the present invention are derived from human monocyte conditioned medium where the monocytes are grown on IgG coated vessels. In addition, the invention encompasses substantially purified IL-1 inhibitors of any origin which are biologically equivalent to the inhibitor derived from human monocyte-contained medium.

By "biologically equivalent," as used throughout the specification and claims, we mean compositions of the present invention that are capable of preventing IL-1 action in a similar fashion, but not necessarily to the safe degree, as the native IL-1 inhibitor isolated from monocytes By "substantially homologous" as used throughout the ensuing specification and claims, is meant a degree of homology to the native IL-1 inhibitor isolated from monocyte-conditioned medium in excess of that displayed by any previously reported IL-1 inhibitors. Preferably, the degree of homology in excess of 70 percent, more preferably in excess of 80 percent and even more preferably in excess of 90 per cent. A particularly preferred group of inhibitors are in excess of 95 percent homologous with the native inhibitor. The percentage of homology as described is calculated as the percentage of amino acid residues found in the smaller of the two sequences that align with identical amino acid residues in the sequence being compared when four gaps in a length of 100 amino acids may be introduced to assist in that alignment as set forth by Dayhoff, M. D. in Atlas of Protein Sequence and Structure Vol.5, p. 124 (1972), National Biochemical Research Foundation, Washington, D.C., specifically incorporated herein by reference.

The preferred IL-1 inhibitors of the present invention have been derived from monocyte-conditioned medium and, for the first time, have been isolated in a purified form. For the purposes of the present application, "pure form" or "purified form" when used to refer to the IL-1 inhibitors disclosed herein, shall mean a preparation which is substantially free of other proteins which are not IL-1 inhibitor proteins. Preferrably, the IL-1 inhibitors of the present invention are at least 90% pure and preferably 95% pure.

At least three purified IL-1 inhibitors have been isolated by the methods of the Example. These include inhibitor 1, inhibitor 2 and inhibitor 3. Inhibitor 1 is behaving as a 22-23 kDa

molecule on SDS-PAGE with an approximate isoelectric point of 4.8 and eluting from a Mono Q FPLC column at around 52 mM NaCl in Tris buffer, pH 7.6. Inhibitor 2 is also a 22-23 kDa protein, pI = 4.8, but eluting from a Mono Q column at 60 mM NaCl. Inhibitor 3 is a 20 kDa protein and elutes from a Mono Q column at 48 mM NaCl. Inhibitors 1, 2 and 3 are related immunologically and functionally. Having obtained these inhibitors in purified forms has enabled the present inventors to obtain their amino acid sequences. Using the purified inhibitors disclosed for the first time herein and methods such as those described in and by ABI Protein Sequencer technical manuals supplied with the ABI Protein Sequencer, a substantial proportion of the amino acid sequences of these inhibitors can be deduced.

Example 3 shows amino acid sequence data obtained of three species of IL-1 inhibitors, namely IL-I i -X, IL-I i - alpha and IL-I i - beta .

The present inventors have discovered at least one antibody raised against an IL-1 inhibitor. Other polyclonal and monoclonal antibodies against this and other IL-1 inhibitors may be prepared by methods known to those of ordinary skill in the art. One particular polyclonal antibody is described in Example 4.
B. Recombinant Inhibitor
1. General

A recombinant DNA method for the manufacture of an IL-1 inhibitor is now disclosed. In one embodiment of the invention, the active site functions in a manner biologically equivalent to that of the native IL-1 inhibitor isolated from human. A natural or synthetic DNA sequence may be used to direct production of the IL-1 inhibitors. This method comprises:

(a) Preparation of a DNA sequence capable of directing a host cell to produce a protein having IL-1 inhibitor activity;

(b) Cloning the DNA sequence into a vector capable of being transferred into and replicated in a host cell, such vector containing operational elements needed to express the DNA sequence;

(c) Transferring the vector containing the synthetic DNA sequence and operational elements into a host cell capable of expressing the DNA encoding IL-1 inhibitor;

(d) Culturing the host cells under conditions appropriate for amplification of the vector and expression of the inhibitor;

(e) Harvesting the inhibitor; and

(f) Permitting the inhibitor to assume an active tertiary structure whereby it possesses IL-1 inhibitory activity.
2. DNA Sequences

DNA sequences contemplated for use in this method are discussed in part in Example 5 and in part in Example 6. It is contemplated that these sequences include synthetic and natural DNA sequences. The natural sequences further include cDNA or genomic DNA segments.

Example 6 provides a molecular clone of DNA encoding a protein identical to that isolated in Examples 1-3. In Example 6, a plaque, GT10-ILli-2A, was isolated from a GT10 Library. The phage within this plaque was propagated and the DNA was isolated and digested with EcoRI. An EcoRI fragment of 1850 base pairs carries the coding sequence for ILI inhibitor. FIG. 13 shows the partial DNA sequence of the EcoRI fragment.

In light of the teachings contained herein and procedures known, other synthetic polynucleotide sequences will be available to one of ordinary skill in the art. As an example of

the current state of the art relating to polynucleotide synthesis, one is directed to Matteucci, M. D. and Caruthers, M. H., in J. Am. Chem. Soc. 103:3185 (1981) and Beaucage, S. L. and Caruthers, M. H. in Tetrahedron Lett. 22:1859 (1981), and to the instructions supplied with an ABI oligonucleotide synthesizer, each of which is specifically incorporated herein by reference.

These synthetic sequences may be identical to the natural sequences described in more detail below or they may contain different nucleotides. In one embodiment, if the synthetic sequences contain nucleotides different from those found in the natural DNA sequences of this invention, it is contemplated that these different sequences will still encode a polypeptide which has the same primary structure as IL-li isolated from monocytes. In an alternate embodiment, the synthetic sequence containing different nucleotides will encode a polypeptide which has the same biological activity as the IL-li described herein.

Additionally, the DNA sequence may be a fragment of a natural sequence, i.e., a fragment of a polynucleotide which occurred in nature and which has been isolated and purified for the first time by the present inventors. In one embodiment, the DNA sequence is a restriction fragment isolated from a cDNA library.

In an alternative embodiment, the DNA sequence is isolated from a human genomic library. An example of such a library useful in this embodiment is set forth by Lawn et al. in Cell 15:1157-1174 (1978), specifically incorporated herein by reference.

In a preferred version of this embodiment, it is contemplated that the natural DNA sequence will be obtained by a method comprising:

(a) Preparation of a human cDNA library from cells, preferably monocytes, capable of generating an IL-1 inhibitor in a vector and cell capable of amplifying and expressing all or part of that cDNA;

(b) Probing the human DNA library with at least one probe capable of binding to the IL-1 inhibitor gene or its protein product;

(c) Identifying at least one clone containing the gene coding for the inhibitor by virtue of the ability of the clone to bind at least one probe for the gene or its protein product;

(d) Isolation of the gene or portion of the gene coding for the inhibitor from the clone or clones chosen;

(e) Linking the gene, or suitable fragments thereof, to operational elements necessary to maintain and express the gene in a host cell.

The natural DNA sequences useful in the foregoing process may also be identified and isolated through a method comprising:

(a) Preparation of a human genomic DNA library, preferably propagated in a recArecBC E. coli host;

(b) Probing the human genomic DNA library with at least one probe capable of binding to an IL-1 inhibitor gene or its protein product;

(c) Identification of at least one clone containing the gene coding for the inhibitor by virtue of the ability of the clone to bind at least one probe for the gene or its protein product;

(d) Isolation of the gene coding for the inhibitor from the clone(s) identified; and

(e) Linking the gene, or suitable fragments thereof, to operational elements necessary to maintain and express the gene in a host cell.

In isolating a natural DNA sequence suitable for use in the above-method, it is preferred to identify the two restriction sites located within and closest to the end portions of the appropriate gene or sections of the gene. The DNA segment containing the appropriate gene is then removed from the remainder of the genomic material using appropriate restriction endonucleases. After excision, the 3' and 5' ends of the DNA sequence and any exxon junctions are reconstructed to provide appropriate DNA sequences capable of coding for the N- and C- termini of the IL-1 inhibitor protein and capable of fusing the DNA sequence to its operational elements.

3. Vectors

(a) Microorganisms, especially E. coli

The vectors contemplated for use in the present invention include any vectors into which a DNA sequence as discussed above can be inserted, along with any preferred or required operational elements, and which vector can then be subsequently transferred into a host cell and replicated in such cell. Preferred vectors are those whose restriction sites have been well documented and which contain the operational elements preferred or required for transcription of the DNA sequence. However, certain embodiments of the present invention are also envisioned which employ currently undiscovered vectors which would contain one or more of the cDNA sequences described herein. In particular, it is preferred that all of these vectors have some or all of the following characteristics: (1) possess a minimal number of host-organism sequences; (2) be stably maintained and propagated in the desired host; (3) be capable of being present in a high copy number in the desired host; (4) possess a regulatable promoter positioned so as to promote transcription of the gene of interest; (5) have at least one marker DNA sequence coding for a selectable trait present on a portion of the plasmid separated from that where the DNA sequence will be inserted; and (6) a DNA sequence capable of terminating transcription.

In various preferred embodiments, these cloning vectors containing and capable of expressing the DNA sequences of the present invention contain various operational elements. These "operational elements," as discussed herein, include at least one promoter, at least one Shine-Dalgarno sequence and initiator codon, and at least one terminator codon. Preferably, these "operational elements" also include at least one operator, at least one leader sequence for proteins to be expoited from intracellular space, at least one gene for a regulator protein, and any other DNA sequences necessary or preferred for appropriate transcription and subsequence translation of the vector DNA.

Certain of these operational elements may be present in each of the preferred vectors of the present invention It is contemplated that any additional operational elements which may be required may be added to these vectors using methods known to those of ordinary skill in the art, particularly in light of the teachings herein.

In practice, it is possible to construct each of these vectors in a way that allows them to be easily isolated, assembled and interchanged. This facilitates assembly of numerous functional genes from combinations of these elements and the coding region of the DNA sequences. Further, many of these elements will be applicable in more than one host. It is additionally contemplated that the vectors, in certain preferred embodiments, will contain DNA sequences capable of functioning as regulators ("operators"), and other DNA sequences capable of coding for regulator proteins.

(i) Regulators

These regulators, in one embodiment, will serve to prevent expression of the DNA sequence in the presence of certain environmental conditions and, in the presence of other environmental conditions, will allow transcription and subsequent expression of the protein

coded for by the DNA sequence. In particular, it is preferred that regulatory segments be inserted into the vector such that expression of the DNA sequence will not occur, or will occur to a greatly reduced extent, in the absence of, for example, isopropylthio-beta-D-galactoside. In this situation, the transformed microorganisms containing the DNA sequence may be grown to at a desired density prior to initiation of the expression of IL-li. In this embodiment, expression of the desired protein is induced by addition of a substance to the microbial environment capable of causing expression of the DNA sequence after the desired density has been achieved.

(ii) Promoters

The expression vectors must contain promoters which can be used by the host organism for expression of its own proteins. While the lactose promoter system is commonly used, other microbial promoters have been isolated and characterized, enabling one skilled in the art to use them for expression of the recombinant IL-li.

(iii) Transcription Terminator

The transcription terminators contemplated herein serve to stabilize the vector. In particular, those sequences as described by Rosenberg, M. and Court, D., in Ann. Rev. Genet. 13:319-353 (1979), specifically incorporated herein by reference, are contemplated for use in the present invention.

(iv) Non-Translated Sequence

It is noted that, in the preferred embodiment, it may also be desirable to reconstruct the 3' or 5' end of the coding region to allow incorporation of 3' or 5' non-translated sequences into the gene transcript. Included among these non-translated sequences are those which stabilize the mDNA as they are identified by Schmeissner, U., McKenney, K., Rosenberg, M and Court, D. in J. Mol. Biol. 176:39-53 (1984), specifically incorporated herein by reference.

(v) Ribosome Binding Sites

The microbial expression of foreign proteins requires certain operational elements which include, but are not limited to, ribosome binding sites. A ribosome binding site is a sequence which a ribosome recognizes and binds to in the initiation of protein synthesis as set forth in Gold, L., et al., Ann. Rev. Microbio. 35:557-580; or Marquis, D. M., et al., Gene 42:175-183 (1986), both of which are specifically incorporated herein by reference. A preferred ribosome binding site is GAGGCGCAAAAA(ATG).

(vi) Leader Sequence and Translational Coupler

Additionally, it is preferred that DNA coding for an appropriate secretory leader (signal) sequence be present at the 5' end of the DNA sequence as set forth by Watson, M. E. in Nucleic Acids Res. 12:5145-5163, specifically incorporated herein by reference, if the protein is to be excreted from the cytoplasm. The DNA for the leader be in a position which allows the production of a fusion protein in which the leader sequence is immediately adjacent to and covalently joined to the inhibitor, i.e., there must be no transcription or translation termination signals between the two DNA coding sequences. The presence of the leader sequence is desired in part for one or more of the following reasons. First, the presence of the leader sequence may facilitate host processing of the IL-li. In particular, the leader sequence may direct cleavage of the initial translation product by a leader peptidase to remove the leader sequence and leave a polypeptide with the amino acid sequence which has potential protein activity. Second, the presence of the leader sequence may facilitate purification of the IL-li, through directing the protein out of the cell cytoplasm. In some species of host microorganisms, the presence of an appropriate leader sequence will allow transport of the completed protein into the periplasmic space, as in the case of some E. coli. In the case of certain E. coli, Saccharomyces and strains of Bacillus and Pseudomonas, the appropriate leader sequence will allow transport of the protein through the cell membrane and into the extracellular medium. In this situation, the protein may be purified from

extracellular protein. Thirdly, in the case of some of the proteins prepared by the present invention, the presence of the leader sequence may be necessary to locate the completed protein in an environment where it may fold to assume its active structure, which structure possesses the appropriate protein activity.

In one preferred embodiment of the present invention, an additional DNA sequence is located immediately preceding the DNA sequence which codes for the IL-1 inhibitor. The additional DNA sequence is capable of functioning as a translational coupler, i.e., it is a DNA sequence that encodes an RNA which serves to position ribosomes immediately adjacent to the ribosome binding site of the inhibitor RNA with which it is contiguous. In one embodiment of the present invention, the translational coupler may be derived using the DNA sequence

TAACGAGGCGCAAAAAATGAAAAAGACAGCTATCGCGATCTTGGAGGATGATTAAATG

and methods currently known to those of ordinary skill in the art related to translational couplers.
(vii) Translation Terminator

The translation terminators contemplated herein serve to stop the translation of mRNA. They may be either natural, as described by Kohli, J., Mol. Gen. Genet. 182:430-439; or synthesized, as described by Pettersson, R. F. Gene 24:15-27 (1983), both of which references are specifically incorporated herein by reference.
(viii) Selectable Marker

Additionally, it is preferred that the cloning vector contain a selectable marker, such as a drug resistance marker or other marker which causes expression of a selectable trait by the host microorganism. In one embodiment of the present invention, the gene for ampicillin resistance is included in the vector while, in other plasmids, the gene for tetracycline resistance or the gene for chloramphenicol resistance is included.

Such a drug resistance or other selectable marker is intended in part to facilitate in the selection of transformants. Additionally, the presence of such a selectable marker in the cloning vector may be of use in keeping contaminating microorganisms from multiplying in the culture medium. In this embodiment, a pure culture of the transformed host microorganisms would be obtained by culturing the microorganisms under conditions which require the induced phenotype for survival.

The operational elements as discussed herein are routinely selected by those of ordinary skill in the ar in light of prior literature and the teachings contained herein. General examples of these operational elements are set forth in B. Lewin, Genes, Wiley & Sons, New York (1983), which is specifically incorporated herein by reference Various examples of suitable operational elements may be found on the vectors discussed above and may be elucidated through review of the publications discussing the basic characteristics of the aforementioned vectors.

Upon synthesis and isolation of all necessary and desired component parts of the above-discussed vector, the vector is assembled by methods generally known to those of ordinary skill in the art. Assembly of such vectors is believed to be within the duties and tasks performed by those with ordinary skill in the art and, as such, is capable of being performed without undue experimentation. For example, similar DNA sequences have been ligated into appropriate cloning vectors, as set forth by Maniatis et al. in Molecular Cloning, Cold Spring Harbor Laboratories (1984), which is specifically incorporated herein by reference.

In construction of the cloning vectors of the present invention, it should additionally be noted that multiple copies of the DNA sequence and its attendant operational elements may be inserted into each vector. In such an embodiment, the host organism would produce greater

amounts per vector of the desired IL-1 inhibitor. The number of multiple copies of the DNA sequence which may be inserted into the vector is limited only by the ability of the resultant vector, due to its size, to be transferred into and replicated and transcribed in an appropriate host cell.

(b) Other Microorganisms

Vectors suitable for use in microorganisms other than E. coli are also contemplated for this invention. Such vectors are described in Table 1. In addition, certain preferred vectors are discussed below.

TABLE 1

HOSTS	REGULATED PROMOTERS	INDUCER	TRANS-CRIPTION TER-MINATOR	MRNA STABILIZATION	TRANSCRIP-TIONAL START SITE & LEADER PEPTIDE	MARKER	RS BINDING SITE
E. coli	Lac<1> , Tac<2>	IPTG	rrnB<6>	ompA<8>	bla<11>	ampicillin<14>	
	Lambda pL	increased	rrnC<7>		lambda int<9> ompA<12>	tetracycline<14, 15>	
	Trp<5>	temperature *			trp<10> phoS	chloramphenical<1	
	*	IAA addition					
	*	or					
	*	tryptophan					
		depletion					
Bacillus	*alpha	*	E. coli rrn	*	B. amy neutral	Kan<r 24>	
	amylase<17>	*	rrn BT.T<20>	*	protease<21>	Cam<r 25>	
	*subtilisin<18>	*	*	*	B. amy alpha-	*	
	*p-43<19>	*	*	*	amylase<22>	*	
	spac-I<26>	IPTG	*	*	B. subt.		
	*	*	*	*	subtilisin<23>		
Pseudo- monas	Trp<27 > (E. coli) Lac (E. coli) * Tac (E. coli)	IAA addition, or tryptophan depletion IPTG	* *	* *	phospholipase C<28> exotoxin A<29>	sulfonamide<30> streptomycin<30>	Trp (E. coli)
Yeast	Gal 1<31> ,	Glucose	Cyc 1	*	Invertase<36>	Ura 3<37>	
	10<32> *	depletion and	Una *	* *	Acid phospha- tase<36>	Leu 2<38>	
	Adh I<33> ,	galactose	Alpha factor	*	Alpha Factor	His 3	

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II<34>    Glucose    Sac 2  *       *              Tap 1
Pho 5     depletion
*         Phosphate
*         depletion
```

n*non-regulated -

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(i) Pseudomonas Vectors

Several vector plasmids which autonomously replicate in a broad range of Gram negative bacteria are preferred for use as cloning vehicles in hosts of the genus Pseudomonas. Certain of these are described by Tait, R. C., Close, T. J., Lundquist, R. C., Hagiya, M., Rodriguez, R. L., and Kado, C. I. In Biotechnology, May, 1983, pp. 269-275; Panopoulos, N. J. in Genetic

Engineering in the Plant Sciences, Praeger Publishers, New York, N.Y., pp. 163-185 (1981); and Sakagucki, K. in Current Topic in Microbiology and Immunology 96:31-45 (1982), each of which is specifically incorporated herein by reference.

One particularly preferred construction would employ the plasmid RSF1010 and derivatives thereof as described by Bagdasarian, M., Bagdasarian, M. M., Coleman, S., and Timmis, K. N. in Plasmids of Medical, Environmental and Commercial Importance, Timmis, K. N. and Puhler, A. eds., Elsevier/North Holland Biomedical Press (1979), specifically incorporated herein by reference. The advantages of RSF1010 are that it is relatively a small, high copy number plasmid which is readily transformed into and stably maintained in both E. coli and Pseudomonas species. In this system, it would be preferred to use the Tac expression system as described for Escherichia, since it appears that the E. coli trp promoter is readily recognized by Pseudomonas RNA polymerase as set forth by Sakagucki, K. in Current Topics in Microbiology and Immunology 96:31-45 (1982) and Gray, G. L., MCKeown, K. A., Jones, A. J. S., Seeburg, P. H., and Heyneker, H. L. in Biotechnology, Feb. 1984, pp. 161-165, both of which are specifically incorporated herein by reference. Transcriptional activity may be further maximized by requiring the exchange of the promoter with, e.g., an E. coli or P. aeruginosa trp promoter. Additionally, the lacI gene of E. coli would also be included in the plasmid to effect regulation.

Translation may be coupled to translation initiation for any of the Pseudomonas proteins, as well as to initiation sites for any of the highly expressed proteins of the type chosen to cause intracellular expression of the inhibitor.

In those cases where restriction minus strains of a host Pseudomonas species are not available, transformation efficiency with plasmid constructs isolated from E. coli are poor. Therefore, passage of the Pseudomonas cloning vector through an r - m + strain of another species prior to transformation of the desired host, as set forth in Bagdasarian, M., et al., Plasmids of Medical, Environmental and Commercial Importance, pp. 411-422, Timmis and Puhler eds., Elsevier/North Holland Biomedical Press (1979), specifically incorporated herein by reference, is desired.
(ii) Bacillus Vectors

Furthermore, a preferred expression system in hosts of the genus Bacillus involves using plasmid pUB110 as the cloning vehicle. As in other host vectors system, it is possible in Bacillus to express the IL-li of the present invention as either an intracellular or a secreted protein. The present embodiments include both systems. Shuttle vectors that replicate in both Bacillus and E. coli are available for constructing and testing various genes as described by Dubnau, D , Gryczan, T., Contente, S., and Shivakumar, A. G. in Genetic Engineering, Vol. 2, Setlow and Hollander eds., Plenum Press, New York, N.Y., pp. 115-131 (1980), specifically incorporated herein by reference. For the expression and secretion of the IL-li from B. subtilis, the signal sequence of alpha-amylase is preferably coupled to the coding region for the protein. For synthesis of intracellular inhibitor, the portable DNA sequence will be translationally coupled to the ribosome binding site of the alpha-amylase leader sequence.

Transcription of either of these constructs is preferably directed by the alpha-amylase promoter or a derivative thereof. This derivative contains the RNA polymerase recognition sequence of the native alpha-amylase promoter but incorporates the lac operator region as well. Similar hybrid promoters constructed from the penicillinase gene promoter and the lac operator have been shown to function in Bacillus hosts in a regulatable fashion as set forth by Yansura, D. G. and Henner in Genetics and Biotechnology of Bacilli, Ganesan, A. T. and Hoch, J. A., eds., Academic Press, pp. 249-263 (1984), specifically incorporated by reference. The lacI gene of E. coli would also be included in the plasmid to effect regulation.
(iii) Clostridium Vectors

One preferred construction for expression in Clostridium is in plasmid pJU12, described by

Squires, C. H. et al., in J. Bacteriol. 159:465-471 (1984) and specifically incorporated herein by reference, transformed into C. perfringens by the method of Heefner, D. L. et al., as described in J. Bacteriol. 159:460-464 (1984), specifically incorporated herein by reference. Transcription is directed by the promoter of the tetracycline resistance gene. Translation is coupled to the Shine-Dalgarno sequences of this same tet<r > gene in a manner strictly analogous to the procedures outlined above for vectors suitable for use in other hosts.
(iv) Yeast Vectors

Maintenance of foreign DNA introduced into yeast can be effected in several ways as described by Botstein, D. and Davis, R. W., in The Molecular Biology of the Yeast Saccharomyces, Cold Spring Harbor Laboratory, Strathern, Jones and Broach, eds., pp. 607-636 (1982), specifically incorporated hereby by reference. One preferred expression system for use with host organisms of the genus Saccharomyces harbors the IL-li gene on the 2 micron plasmid. The advantages of the 2 micron circle include relatively high copy number and stability when introduced into ciro strains. These vectors preferably incorporate the replication origin and at least one antibiotic resistance marker from pBR322 to allow replication and selection in E. coli. In addition, the plasmid will preferably have the two micron sequence and the yeast LEU2 gene to serve the same purposes in LEU2 defective mutants of yeast.

If it is contemplated that the recombinant IL-1 inhibitors will ultimately be expressed in yeast, it is preferred that the cloning vector first be transferred into Escherichia coli, where the vector would be allowed to replicate and from which the vector would be obtained and purified after amplification. The vector would then be transferred into the yeast for ultimate expression of the IL-1 inhibitor.
(c) Mammalian Cells

The cDNA for the IL-1 inhibitor will serve as the gene for expression of the inhibitor in mammalian cells. It should have a sequence that will be efficient at binding ribsomes such as that described by [Kozak, in Nucleic Acids Research 15:8125-8132 (1987), specifically incorporated herein by reference,] and should have coding capacity for a leader sequence (see section 3(a)(vi)) to direct the mature protein out of the cell in a processed form. The DNA restriction fragment carrying the complete cDNA sequence can be inserted into an expression vector which has a transcriptional promoter and a transcriptional enhancer as described by Guarente, L. in Cell 52:303-305 (1988) and Kadonaga, J. T. et al., in Cell 51:1079-1090 (1987), both of which are specifically incorporated herein by reference. The promoter may be regulatable as in the plasmid pMSG (Pharmacia Cat. No. 27450601) if constitutive expression of the inhibitor is harmful to cell growth. The vector should have a complete polyadenylation signal as described by Ausubel, F. M. et al. in Current Protocols in Molecular Biology, Wiley (1987), specifically incorporated herein by reference, so that the mRNA transcribed from this vector is processed properly. Finally, the vector will have the replication origin and at least one antibiotic resistance marker from pBR322 to allow replication and selection in E. coli.

In order to select a stable cell line that produces the IL-1 inhibitor, the expression vector can carry the gene for a selectable marker such as a drug resistance marker or carry a complementary gene for a deficient cell line, such as a dihydrofolate reductase (dhfr) gene for transforming a dhfr< - > cell line as described by Ausubel et al., supra. Alternatively, a separate plasmid carrying the selectable marker can be cotransformed along with the expression vector.
4. Host Cells/Transformation

The vector thus obtained is transferred into an appropriate host cell. These host cells may be microorganisms or mammalian cells.
(a) Microorganisms

It is believed that any microorganism having the ability to take up exogenous DNA and express those genes and attendant operational elements may be chosen After a host organism has been chosen, the vector is transferred into the host organism using methods generally known to those of ordinary skill in the art. Examples of such methods may be found in Advanced Bacterial Genetics by R. W. Davis et al.. Cold Spring Harbor Press, Cold Spring Harbor, N.Y. (1980). which is specifically incorporated herein by reference. It is preferred, in one embodiment, that the transformation occur at low temperatures, as temperature regulation is contemplated as a means of regulating gene expression through the use of operational elements as set forth above In another embodiment, if osmolar regulators have been inserted into the vector, regulation of the salt concentrations during the transformation would be required to insure appropriate control of the foreign genes.

It is preferred that the host microorganism be a facultative anaerobe or an aerobe. Particular hosts which may be preferable for use in this method include yeasts and bacteria. Specific yeasts include those of the genus Saccharomyces, and especially Saccharomyces cerevisiae Specific bacteria include those of the genera Bacillus, Escherichia, and Pseudomonas, especially Bacillus subtilis and Escherichia coli. Additional host cells are listed in Table I, supra.
(b) Mammalian Cells

The vector can be introduced into mammalian cells in culture by several techniques such as calcium phosphate:DNA coprecipitation, electroporation, or protoplast fusion. The preferred method is coprecipitation with calcium phosphate as described by Ausubel et al., supra.

Many stable cell types exist that are transformable and capable of transcribing and translating the cDNA sequence, processing the precursor IL-li and secreting the mature protein. However, cell types may be variable with regard to glycosylation of secreted proteins and post-translational modification of amino acid residues, if any. Thus, the ideal cell types are those that produce a recombinant IL-1 inhibitor identical to the natural molecule.
5. Culturing Engineered Cells

The host cells are cultured under conditions appropriate for the expression of the IL-1 inhibitor These conditions are generally specific for the host cell, and are readily determined by one of ordinary skill in the art in light of the published literature regarding the growth conditions for such cells and the teachings contained herein For example, Bergey's Manual of Determinative Bacteriology, 8th Ed., Williams & Wilkins Company, Baltimore, Md., which is specifically incorporated herein by reference, contains information on conditions for culturing bacteria. Similar information on culturing yeast and mammalian cells may be obtained from Pollack, R. Mammalian Cell Culture, Cold Spring Habor Laboratories (1975), specifically incorporated herein by reference.

Any conditions necessary for the regulation of the expression of the DNA sequence, dependent upon any operational elements inserted into or present in the vector, would be in effect at the transformation and culturing stages. In one embodiment, cells are grown to a high density in the presence of appropriate regulatory conditions which inhibit the expression o(the DNA sequence. When optimal cell density is approached, the environmental conditions are altered to those appropriate for expression of the DNA sequence. It is thus contemplated that the production of the IL-1 inhibitor will occur in a time span subsequent to the growth of the host cells to near optimal density, and that the resultant IL-1 inhibitor will be harvested at some time after the regulatory conditions necessary for its expression were induced.
6. Purification
(a) IL-li Produced From Microorganisms

In a preferred embodiment of the present invention, the recombinant IL-1 inhibitor is purified subsequent to harvesting and prior to assumption of its active structure. This embodiment is preferred as the inventors believe that recovery of a high yield of re-folded protein is

facilitated if the protein is first purified. However, in one preferred, alternate embodiment, the IL-1 inhibitor may be allowed re-fold to assume its active structure prior to purification. In yet another preferred., alternate embodiment, the IL-1 inhibitor is present in its re folded, active state upon recovery from the culturing medium.

In certain circumstances, the IL-1 inhibitor will assume its proper, active structure upon expression in the host microorganism and transport of the protein through the cell wall or membrane or into the periplasmic space. This will generally occur if DNA coding for an appropriate leader sequence has been linked to the DNA coding for the recombinant protein. If the IL-1 inhibitor does not assume its proper, active structure, any disulfide bonds which have formed and/or any noncovalent interactions which have occurred will first be disrupted by denaturing and reducing agents, for example, guanidinium chloride and beta-mercaptoethanol, before the IL-1 inhibitor is allowed to assume its active structure following dilution and oxidation of these agents under controlled conditions.

For purification prior to and after refolding, some combination of the following steps is preferably used: anion exchange chromatography (MonoQ or DEAE-Sepharose), gel filtration chromatography (superose), chromatofocusing (MonoP), and hydrophobic interaction chromatography (octyl or phenyl sepharose). Of particular value will be antibody affinity chromatography using the IL-li-specific monoclonal antibodies (described in Example 3).
(b) IL-li Produced from Mammalian Cells

IL-li produced from mammalian cells will be purified from conditioned medium by steps that will include ion exchange chromatography and immunoaffinity chromatography using monoclonal antibodies described in Example 3. It will be apparent to those skilled in the art that various modifications and variations can be made in the processes and products of the present invention. Thus, it is intended that the present invention cover the modifications and variations of this invention provided they come within the scope of the appended claims and their equivalents.

It is to be understood that application of the teachings of the present invention to a specific problem or environment will be within the capabilities of one having ordinary skill in the art in light of the teachings contained herein. Examples of the products of the present invention and representative processes for their isolation and manufacture appear in the following.

The following examples illustrate various presently preferred embodiments of the present invention. The publications provided in this examples are specifically incorporated by reference herein.
EXAMPLES
Example 1
Protein Preparation
A. Materials

Hank's Balanced Salt Solution (HBSS) and RPMI were purchased from Mediatech, Washington, D.C. Lymphoprep was obtained from Accurate Chemical and Scientific Corp., Westbury, N.Y. Human IgG, MTT, rabbit anti-prostaglandin E2 antiserum, ammonium bicarbonate, dithiothreitol, complete and incomplete Freund's adjuvants, hypoxanthine, aminopterin, and thymidine were purchased from Sigma Chemical Co., St. Louis, Missouri. C3H/HeJ mice were purchased from Jackson Labs, Bar Harbor, Me. BALB/c mice and P3 myeloma cells were obtained from Drs. John Kappler and Philippa Marrack at the National Jewish Center for Immunology and Respiratory Medicine (NJC/IRM), Denver, Colo. Recombinant human IL-1 was obtained from Cistron Biotechnology, Pine Brook, N.J purified phytohemagglutinin was purchased from Wellcome Diagnostics, Research Triangle Park, N.C. Human foreskin fibroblasts from primary cultures were obtained from Dr. Richard Clark at the NJC/IRM, Denver, Colorado. Monoclonal mouse anti-rabbitt IgG antibodies were purchased from AIA reagents, Aurora, Colo. Low methionine RPMI was made using a Select-Amine kit

from GIBCO Laboratories, Grand Island, N.Y. [<35> S]-methionine, diphenyloxazole, and [<14> C]-iodoacetic acid were obtained from DuPont NEN, Chicago, Ill. Fetal calf serum was purchased from HyClone Laboratories, Logan, Utah. Mono Q and Superose 12 columns were purchased from Pharmacia, Inc., Piscataway, N.J. C4-reversed phase columns were obtained from Synchrom, Inc., Lafayette, Ind. C8-reversed phase columns were obtained from Applied Biosystems, Inc., Foster City, Calif. Acetonitrile and polyethylene glycol 8000 were purchased from J. T. Baker Chemical Co., Phillipsburg, N.J. Trifluroacetic acid and guanidine hydrochloride were obtained from Pierce Chemicals, Rockford, Ill. Endoproteinase Lys C was obtained from Boehringer Mannheim Biochemicals, Indianapolis, Ind. The microtitering plates used for PGE2 ELISA were Nunc-Immuno Plate I obtained from Intermountain Scientific Corporation, Bountiful, Utah. The plates used for hybridoma production were from Costar, Cambridge, Mass.

B. Generation of Monocyte IL-1 Inhibitor

Human leukocytes were obtained from normal donors by leukophoresis, resuspended in Hank's balanced salt solution (HBAA) at 1 part packed cells to 1 part HBSS, underlayed with Lymphoprep and spun at 400 xg for 30' at room temperature. The mononuclear fraction was taken (typically 4- x 10<9 > cells were obtained per donor), washed in HBSS without Ca< + + > or Mg< + + > , suspended in serum-free RPMI and plated on petri dishes coated with normal human IgG made LPS free by chromatography over Sephapex G200 (6 x 10<7 > cells in 10 ml per 100 mm dish). All reagents contained less than 10 pg/ml LPS. The cells were cultured 24-48 hr, and the resulting conditioned medium constituted the crude IL-1 inhibitor (IL-li) supernatant. Typically, the cells from one donor yielded 700-900 ml crude IL-li supernatant.

C. Assays for the IL-1 Inhibitor

Two IL-1 assays have been used routinely to detect the IL-li. Thymocytes (1 x 10<6 > cells from 4 to 6 week old C3H/HeJ mice) respond to 1.0 unit/ml of recombinant human IL-1 plus 1 ug/ml phytohaemaglutinin by proliferatirg half-maximally, as measured by 3H-thymidine incorporation or uptake of the tetrazolium salt MTT (Mosmann, T., J. Immunol Method, 65:55-61 (1983)) after three days of stimulation. Crude IL-li supernatant fully inhibits this proliferative response at a 1/13 dilution. Human dermal fibroblasts (1 x 10<5 > cells per well in a 96 well plate) typically respond to 0.5 units/ml recombinant human IL-1 by secreting, at 6 hours of stimulation, approximately 50,000 pg/ml PGE2 that can be measured by ELISA. This assay is as sensitive to IL-li as is the thymocyte assay.

D. Metabolic Labeling of the IL-1 Inhibitor

The IL-li was metabolically labeled by culturing mononuclear leukocytes for 48 hours on IgG-coated plates (as described in B) in serum-free RPMI containing only 0.75 ug/ml cold methionine (15 ug/ml is normal) and to which was added 0.5 mCi <35> S-methionine (1151 Ci/mmol) per 10<7 > cells. Control labelings were performed serum rather than IgG. Assays on such control supernatants showed that very little IL-li was secreted when the cells were cultured on on fetal calf serum-coated plates.

E. Purification of the IL-1 Inhibitor Protein

Crude IL-li supernatants were made 1.0 M in sodium chloride, incubated on ice for 1 hour and centrifuged at 10,000 rpm for 15 minutes. The supernatants, which contained all of the inhibitor activity but only 20% of the initial protein, were then dialyzed extensively at 4o C. versus 0.025 M Tris, pH 7.6 containing 0.1% sucrose (the A buffer) for gradient fractionation of proteins on a Mono Q anion exchange column. Following dialysis the inhibitor-containing solutions were recentrifuged at 10,000 rpm for 15 minutes and then passed through 0.22 u nylon filters. The supernatants were typically combined with 10 ml of similarly prepared supernatant from a metabolic labeling and loaded onto Mono Q-Superose (Pharmacia FPLC) columns with bed volumes of either 1.0 ml or 8.0 ml, washed with A buffer until the OD280 of the effluent returned to baseline, and carefully chromatographed using a linear sodium chloride gradient (0.025 M to 0.10 M) in buffer A. Column fractions were collected and

analyzed for radioactivity and bioactivity. Samples of each fraction were also run on reduced 12.5% SDS-PAGE, silver stained, permeated with diphenyloxazole, dried and put onto film to obtain autoradiographic data. FIG. 1a shows the protein profile of the Mono Q chromatography of 40 ml crude Il-li supernatant mixed with 3 ml of metabolically labeled IL-li supernatant. Superimposed are the amount of radioactivity found in 50 ul of each fraction as well as the IL-li bioactivity as measured in the PGE2-production assay. Two major and one minor radioactive species are shown that perfectly correlate with three peaks of bioactivity. FIG. 1b shows the similar chromatography of 15 ml of crude Il-li supernatant mixed with 3 ml of supernatant from monocytes metabolically labeled on plates coated with fetal calf serum (FCS) rather than IgG. The levels of the three radioactive species discussed above are markedly diminished. FIG. 2a shows silver stained gels run on the fractions from the regions of interest in the chromatographies shown in FIGS. 1a and 1b. Note that the fractions of peak radioactivity and bioactivity in FIG. 1a (fractions 52 and 59) both show a major band at 22 Kd (marked with arrows) on SDS-PAGE. The third species (fraction 48 in FIG. 1a) shows a band at 20 kD on SDS-PAGE. Gel filtration experiments on crude IL-li have shown that the active molecule has a molecular weight of 18-25 Kd FIG. 2b is an autoradiogram of the gels shown in FIG. 2a. It can be readily seen that the protein bands at 20 and 22 Kd are the major radioactive species in those fractions.

Summarizing these results, we have shown that the metabolic labeling of monocytes plated on petri dishes coated with IgG results in radioactive species that are only poorly produced if the cells are plated on dishes coated with FCS. These induced radioactive species perfectly co-chromatograph with several species of IL-li bioactivity on Mono Q, and gels and resulting autoradiograms show that the three major induced molecules are proteins of the predicted molecular weight for IL-li.

The IL-li molecules were further purified for sequencing in two ways First, Mono Q fractions with peak bioactivity and radioactivity were loaded onto a C4-reversed phase column and eluted with an H2O/0.1% TFA acetonitrile/0.1% TFA gradient Since the IL-li molecule was trace labeled, samples from each fraction were directly counted for radioactivity and were also analyzed by SDS-PAGE followed by autoradiography. FIG. 3a shows such a chromatograph with the radioactivity pattern superimposed. The silver stained gels run on samples from each fraction (FIG. 3b) and subsequent autoradiograms of the gels (FIG. 3c) shows that the IL-li molecule is found in fractions 32-36. These fractions were dried down and sequenced Alternatively, the peak Mono Q fractions were dried by Speed Vac, resuspended in 0.4 ml 0.05 M NH4HCO3 and directly chromatographed two times on a 10 x 300 mm Superose 12 gel filtration column (Pharmacia FPLC) equilibrated in the same buffer, as shown in FIGS. 4a and 4b. Fractions were collected and samples of each were tested for radioactivity and bioactivity and were analyzed by silver stained and autoradiographed SDS-PAGE Appropriate fractions were then dried on a speed vac and sequenced.
Example 2
Proposed Sequencing of the IL-1 Inhibitor

Prior to sequencing, samples were dissolved in 6 M guanidine-HCl, pH 8.6, reduced for 4 hours at 37o C. under N2 with 100-fold molar excess dithiothreitol over protein, and alkylated for 1 hour with 400-fold excess 14C-iodcacetic acid. In that case, the reactions would be desalted on a C8-reversed phase column, eluted, and partially dried. N-terminal sequences will be determined using an Applied Biosystems Protein Sequencer. To obtain internal sequences, samples which may have been reduced and alkylated would be digested with cyanogen bromide or proteolytic enzymes using methods known to those of ordinary skill in the art. Reactions will be dried, dissolved in 0.1% TFA/H2O, and peptides will be separated using a C8-reverse phase column.
Example 3
Purification and Sequencing of the Species of IL-1 Inhibitors
A. IL-li-X, IL-li-a and IL li-h Species

The Mono Q purification of IL-li resolves the biological activity into three major species, as shown in FIG. 1a and described in Example 1, where the peak fractions for this activity are 48, 52, and 59 SDS-PAGE on samples of these fractions, as shown in FIG. 2a, reveal pertinent species at 20 kD, 22 kD, and 22 kD, respectively. Western analysis of such gels, using the mouse antisera discussed in Example 4 below, stains all three of these species. When IL-li is prepared from cells metabolically labeled with <35> S-methionine, during growth on plates coated with IgG, each of these bands is radioactive (as shown in FIG. 2b, the autoradiogram of the above-mentioned gel). Based on the logic discussed in Example 1, namely that parallel cells incubated in a non-inducing condition do not produce the IL-li bioactivity and do not produce these radioactive bands, we can conclude that these three species account for the biological activity. We have tentative-y named these species IL-li-X, IL-li-a, and IL-li-b, respectively.
B Purification and Sequencing of IL-li-X

Mono Q fractions containing IL-li-X and/or IL-li-a were further purified by reversed-phase HPLC chromatography on a Synchropak RP-4 (C4) column, and radioactive species were submitted for sequence analysis. Numerous attempts at directly sequencing RP-HPLC-purified IL-li-a and IL-li-b have failed, suggesting that they are chemically blocked at their N-termini However, one preparation of IL-li-a (IL-li-aB2p42) yielded the following sequence: [See Original Patent for Chemical Structure Diagram]

and subsequent preparations of IL-li-X, similarly purified by C4 RP-HPLC, have produced the same sequence: [See Original Patent for Chemical Structure Diagram]

These are obviously part of the sequence found in the initial attempt at sequencing IL-li-a. It is the inventors' conclusion that the sequence data shown is the N-terminus of the 20 kD species called IL-li-X.

In these and all subsequent sequences an underlined position indicates either an inability to identify a residue or that ambiguity exists with respect to the residue identified. When two or more residues are put in one position, it indicates that more than one amino acid was detected at that sequencing step, and the more likely correct residue is on top.
C. Generation, Purification, and Sequencing of peptides of IL-li-a and IL-li-b

Since IL-li-a and IL-li-b are apparently chemically blocked at their N-termini, peptides of each were generated by endoproteinase digestion Specifically, Mono Q fractions containing either IL-li-a or IL-li-b were passed through a 4.6 x 250 C3-RPHPLC column (Zorbax Protein Plus., an acceptable alternative to the C-4 columns used in all previous experiments. Very gradual gradients (0.2% acetonitrile per minute at 0.5 ml/min) resolved the IL-li-a (FIG. 8a,b) or IL-li-b (FIG. 9a) away from the major contaminating radioactive species, human lysozyme. The identities of the purified species were confirmed by the presence of a single, radioactive, 22 kD protein on SDS-pAGE and subsequent autoradiograms (FIGS. 8c,d and 9b). The proteins were hand-collected into siliconized glass tubes and to each was added 25 ml of a 0.2% Tween-20 solution. The IL-li-containing fractions were then reduced in volume on a Speed-Vac to 50 ml, brought up to 300 ml by the addition of 1% NH4HCO3, followed by the addition of 1 mg of endoproteinase. In the case of IL-li-a, the enzyme used was Endoproteinase Lys C (Boehringer-Mannheim), while IL-li-b was cleaved with Endoproteinase Asp N (Boehringer-Mannheim). Cleavage was carried out at 37o C. for 16 hr, and then the volume of the reaction mix was reduced to 50 ml on a Speed Vac.

In the case of IL-li-a, the sample was directly chromatographed, whereas the IL-li-b sample was first reduced by the addition of 5 ml of 50 mM dithiothreitol in 2 M Tris, pH 8.0, reacted for 30 min at 37o C., and then carboxymethylated by addition of 1.1 umole <3> H-iodoacetic acid in 10 ml ethanol (reacted 30 min at 37o C. in the dark) Separation of the peptides was

performed on a 2.1 x 250 mm Brownlee Aquapore RP-300 (C8) narrow-bore column at a flow rate of 100 ml/min using a Beckman HPLC outfitted with microbore hardware and microbore-compatible pumps A 200 min 0-100% linear gradient was used (H2O/0.1% TFA to acetonitrile/0.1% TFA). The peptide separations are shown in FIGS. 10 and 11. The sequence information obtained is as follows: [See Original Patent for Chemical Structure Diagram]

Two of the peptide sequences are obviously related to that which was obtained earlier from IL-li-X. One of these, RaLysC-41, is an IL-li-a sequence, and the other, RbAspN-51, is an IL-li-b sequence, arguing that the three species of Il-li are at least closely related proteins if not chemically and/or physically modified forms of a single original IL-li molecule. If the listed sequences are combined, the following composite sequences result: [See Original Patent for Chemical Structure Diagram]

These composite sequences appear to be present in no other known polypeptides listed in the most recently updated Protein Identification Resource Database (PIR 16.0). The inventors believe that these sequences, or minor variants thereof, represent a class of molecules that are capable of acting as IL-1 inhibitors.
Example 4
Preparation of Antibodies Specific For the IL-1 Inhibitor

Ten week old BALB/c mice were injected subcutaneously with IL-li that was partially purified (400-fold) from crude supernatants by Mono Q-chromatography, dialyzed versus PBS, and emulsified with Complete Freund's Adjuvant. Each mouse received the IL-li purified from 5 ml of crude supernatant. The mice were boosted every two weeks with an equivalent amount of IL-li emulsifed with Incomplete Freund's Adjuvant, and serum samples were taken from the tails seven days after each boost. Antisera were tested for anti-IL-li activity by Western analysis of transblots of the immunogen run on SDS-PAGE, as shown in FIG. 5a. FIG. 5b shows that all of the mice were making anti-IL-li antibodies after three injections of IL-li.

Since monoclonal antibodies will be of great value in cloning the IL-li gene from an expression library, purifying the recombinant IL-li protein, and studying the biology of the molecule, we have begun the process of making a battery of monoclonal antibodies specific for Il-li. To produce B cell hybridomas, the above mice were injected intravenously with the same amount of IL-li in saline 24 hours prior to removal of the spleens. Splenocytes were teased out of the spleens into cold balanced salt solution (BSS), washed two times with BSS, mixed with P3 myeloma cells at a ratio of 2 x 10<7 > P3 cells per 10<8 > splenic B cells and spun down. The cells were fused by the dropwise addition of 1 ml of warm, gassed (5% CO2) PEG 6000 (40% polyethylene glycol 6000:60% minimal essential medium) to the dry pellet. Fused cells were washed with BSS and resuspended in 10 ml of rich media (10% FBS) containing 2 x 10<5 > peritoneal cells per ml and the pellet was gently broken up using a 10 ml pipet. The volume was adjusted to 20 ml with the addition of more peritoneal cells in media, and the cells were plated out in 96 well plates at 0.1 ml/well. Plates were placed in a gas incubater and treated in the following manner thereafter:

Day 1-Add 3 x HAT (hypoxanthine, aminopterin, thymidine) in rich medium to a final concentration of 1 x

Day 5-Change medium, replacing with 200 ul 1 x HAT in rich medium

Day 10-Begin checking for hybrid growth. Change medium, replacing with 200 ul 1 x HAT in rich medium containing 1.5 x 10<6 > peritoneal cells per ml.

When hybrid cells are nearly confluent in a well the supernatants are transfered for testing, and the cells are gently scraped with a pipet tip and transfered to 1 ml culture wells containing 1 x HAT in rich medium plus 3 x 10<6 > peritoneal cells per ml.

The supernatants from the confluent wells are tested for anti-IL-li activity using an ELISA in which partially purified IL-li (Mono Q-purified material identical to that injected into the mice) is bound to microtitering wells. Normal mouse sera and hyperimmune antisera are used as the negative and positive controls, respectively. Positive supernatants will be retested by ELISA on plates coated with homogeneously purified IL-li and by immunoprecipitation of purified metabolically labeled IL-li. Positive cells will then be cloned by limiting dilution and injected into pristane-treated mice for the generation of ascites. Large quantities of IL-li-specific antibodies can be produced by tissue culture or by massive generation and collection of ascitic fluid in mice. Purification of these antibodies and attachment thereof to insoluble beads will produce affinity adsorbents for the purification of the recombinant IL-li protein.
Example 5
Cloning the Il-li cDNA

It was shown that monocytes plated on IgG-coated petri dishes and cultured for 24 hours in the presence of [<35> S]-methionine produced [<35> S]-IL-li which could be identified by its chromotographic properties on Mono Q.

In order to determine when (during the 24 hour period) IL-li was being produced at a maximal rate, plated monocytes were exposed to [<35> S]-methionine (pulsed) for a short, two-hour period, at which time a large excess of unlabelled methionine was added and incubated for an additional two hours. The medium was then collected and analyzed for ratiolabelled IL-li. This procedure was applied to monocytes at various times after plating of IgG-coated plates and it was found that exposing monocytes to [<35> S]-methionine at 15 hours after plating produced the maximal amount of [<35> S]-IL-li, indicating that IL-li mRNA in monocytes was at its maximal level 15 hours after plating on IgG.

Fresh monocytes were then plated on LPS free IgG obtained as in Example 1B. After incubating in RPMI media for 15 hours at 37o C., the cells are washed with phosphate buffered saline then lysed with 4M guanidinium thiocyanate; 25 mM sodium citrate, pH 7, 0.5% sarcosyl, 0.1 M 2-mercaptoethanol. Total RNA was then isolated from this lysate by the AGPC method of P. Chomczynski and N. Sacchi described in Analytical Biochemistry, vol. 162, pp. 156-159 (1987).

Poly A< + > RNA was isolated by oligo dT cellulose chromatography by the method of Aviv, H. and Leder, P. (1972) Proc. Natl. Acad. Sci. (USA) 69:1408-1412 precipitated with ethanol and dissolved to a concentration of 0.36 ug/ul. One microgram to poly A< + > RNA was used to prepare cDNA according to Gubler, U. and Hoffman, B. J. (1983) Gene 25:263-169.

The cDNA was incorporated into a lambda gtII expression library using Eco RI linkers from Boehringer Mannheim catalog No. 988448 or New England Bio Lab No. 1070 and instructions provided by these manufacturers.

The resulting library, which contains 10<6 > independent clones, was screened on E. coli Y1090 rk< - > (Promega Biotec) with an appropriate polyclonal antibody to IL-li as described previously using screening conditions described by R. A. Young and R. W. Davis [(1983) PNAS 80:1194-1198]. Positive signals will be detected using a biotinylated second antibody (such as goat anti-mouse IgG, Bethesda Research Labs) followed by a strepavidin-alkaline phosphatase conjugate (Bethesda Research Labs), as described by Bayer, E. A. and Wilchek, M. (1979) in Methods in Biochemical Analysis, and Guesdon, J. L. Ternynch, T. and Avrameas, S. (1979) J. Histochem. Cytochem 27:1131-1138 and according to manufacturer's instructions.
Example 6

Preparation and Sequencing of Gene Encoding Il-li

cDNA prepared as described in Example 5 was incorporated into the cloning vector lambda GT10. This cDNA was first methylated using EcoRI methylase with S-adenosyl-methionine as the substrate, EcoRI linkers were attached in a ligation reaction, and excess linkers were removed by digestion with EcoRI endonuclease and chromatography on a CL6B spin column A ligation reaction containing 0.124 ug of linkered, size-selected cDNA and 1 ug of EcoRI-cut and phosphatase treated lambda GT10 was performed, and the products of this ligation reaction were packaged using GIGAPACK GOLD packaging extracts (Stratagene). This yielded a library of 1×10^7 members.

In order to screen this GT10 library, oligonucleotde (antisense) probes were synthesized based on protein and peptide sequence presented in Example 3. The sequences of the probes and of their corresponding peptide sequence are as follows. [See Original Patent for Chemical Structure Diagram]

nNote:

nN = A, G, C, and T

Probe #ILlil-3 was ^{32}P-phosphorylated at its 5' end and used to screen 3×10^5 plaques of the library. The probe hybridized reproducibly to three plaques, and out of these, one plaque was shown to also hybridize to probe #ILlil-4. This plaque, GT10-ILli-2A, was cultivated and the DNA was isolated using Lambdasorb (Promega) according to the manufacturer's instructions. GT10-ILli-2A has been deposited at American Type Culture Collection (ATCC) in Rockville, Md. under Accession No. 40488 The DNA was digested with EcoRI, divided into five equal aliquots, and electrophoresed on a 1% agarose gel.

After electrophoresis, this gel was stained with ethidium bromide. A photograph of this gel is shown in FIG. 12 A. Lanes 6, 8, 10, 12, and 14 contain the five aliquots from the EcoRI digestion. Lane 5 contains a mixture of wild-type lambda DNA cut with HindIII and 0X174 RF DNA cut with HaeIII (New England Biolabs) which are useful as molecular weight markers. FIG. 12a shows that GT10-ILli-2A contains an EcoRI fragment that is 1850 base pairs in length.

In order to demonstrate more conclusively that this 1850 bp fragment carries coding sequence for the ILI inhibitor, a Southern blot was performed as follows. The DNA fragments in the gel shown in FIG. 12A were blotted onto nitrocellulose using standard methods The nitrocellulose was then cut lengthwise into five strips such that each strip contained the DNA from lanes 6, 8, 10, 12, and 14. The strips were then individually hybridized to each of the five oligonucleotide probes (above) which were labeled at the 5' end with ^{32}P phosphate. The oligonucleotide concentration was 1 pmole/ml and the hybridization temperatures were as follows.

LANE	PROBE	TEMPERATURE
6	#ILlil-3	35o C.
8	#ILlil-4	42o C.
10	#ILlil-5	42o C.
12	#ILlil-6	40o C.
14	#ILlil-7	35o C.

After washing, the strips were lined up and taped together to reform the original nitrocellulose sheet. This was autoradiographed in the presence of an intensifying screen at -70o C. for 24 hours. FIG. 12B is a photograph of this autoradiograph. It provides evidence that all of the probes hybridize specifically to the 1850 bp fragment, proving that this fragment carries substantial coding sequences for the ILI inhibitor.

In order to determine its DNA sequence, GT10-ILII-2A DNA was digested with EcoRI, electrophoresed on a 1% agarose gel, and the 1850 bp fragment was isolated. This fragment was ligated with EcoRI-digested M13 mp19 and transformed into E. coli strain JM109. Transformants were screened by looking for those lacking beta-galactosidase activity. Five such transformants were isolated, single-stranded DNA was prepared, and sequencing was performed according to Sanger et al. The DNA sequence of three of the transformants corresponded to the 3' end of the mRNA, while two transformants provided protein coding sequence. In FIG. 13, the DNA sequence is shown that was obtained for the protein coding region of the cDNA.

FIG. 13 also shows the predicted amino acid sequence. The amino acid sequence from the first amino acid Alanine to the 29th amino acid Proline and from the 79th amino acid isoleucine to the end is the hypothesized amino acid sequence. The predicted amino acid sequence from the 30th amino acid Proline to the 78th amino acid Proline agrees with the peptide sequences described in Example 3.
Example 7
Sequencing GT10-IL-II-2A and IL-li

A portion of GT10-ILII-2A has been sequenced and is set forth in FIG. 14. The DNA encodes a protein containing amino acid sequences that are characteristic of IL-li (nucleotides 99-557). However, it is believed that several modifications may be made to this protein before it is secreted into the extracellular milieu. These modifications may or may not be essential for the protein to have activity as an IL-li.

GT10-ILli-2A encodes at least 32 amino acids N-terminal (nucleotides 3-98) to the amino terminus of the form of IL-li known as X. It is believed that included in these 32 amino acids is a secretory leader sequence that starts at the M encoded by nucleotides 24-26, directs the nascent IL-li to the extracellular milieu, and is then removed by a leader peptidase, and possibly other peptidases. The extent to which this sequence is removed in forms alpha and beta of IL-li is presently unknown, but the N-terminus of these forms is thought to be close to that of form X. Removal of the secretory leader sequence is probably required for the protein to have effective IL-li activity.

Nucleotides 349-351 of GT10-ILII-2A encode an N residue that is part of a concensus N-glycosylation site. On the basis of their susceptibility to digestion with N-glycanase it is believed that forms alpha and beta of IL-li are glycosylated. Since form X is not believed to be susceptible to digestion with this enzyme it is believed that it is not glycosylated, although this remains a possibility that could easily be demonstrated by one of ordinary skill in the art of protein sequencing using the information provided here It is believed that glycosylation at this N residue is not required for the protein to show effective IL-li activity.

Nucleotides 99-101 of GT10-ILli-2A encode a P (see FIG. 15), but no P has been detected at this position (the N-terminus) of form X of IL-li. It is possible that this residue has been modified in the mature protein. It is believed that modification of this residue is not essential for effective IL-li activity.

The presently unknown N-terminus residues of forms alpha and beta are not wholly detectable by Edman degradation and are likely to be modified following removal of some of the N-terminal residues of the protein encoded by GT10-ILli-2A. It is believed that is

modification is not essential for effective IL-li activity.
EXAMPLE 8
Expression of Genes Encoding IL-li in Animal Cells

Animal-cell expression of IL-li requires the following steps:

a. Construction of an expression vector

b. Choice of a host cell line

c. Introduction of the expression vector into host cells

d. Manipulation of recombinant host cells to increase expression levels of IL-li

1. IL-li expression vectors designed for use in animal cells can be of several types including strong consitutitve expression constructs, inducible gene constructs, as well as those designed for expression in particular cell types. In all cases promoters and other gene regulatory regions such as enhancers (inducible or not) and polyadenylation signals are placed in the appropriate location in relation to the cDNA sequences in plasmid-based vectors. Two examples of such constructs follow: (1) A construct using a strong constitutive promoter region should be made using the simian virus 40 (SV40) gene control signals in an arrangement such as that found in the plasmid pSV2CAT as described by Gorman et al. in Mol. Cel. Biol. 2:1044-1051, 1982, specifically incorporated herein by reference. This plasmid should be manipulated in such a way as to substitute the IL-li cDNA for the chloramphenicol acetyltransferase (CAT) coding sequences using standard molecular biological techniques (Maniatis et al., supra), as shown in FIG. 6 (2) An inducible gene construct should be made utilizing the plasmid pMK which contains the mouse metallothionein (MT-1) promoter region (Brinster et al., Cell 27:228-231, 1981). This plasmid can be used as a starting material and should be manipulated as shown in FIG. 7 to yield a metal-inducible gene construct.

2. A number of animal cell lines should be used to express IL-li using the vectors described above to produce active protein. Two potential cell lines that have been well-characterized for their ability to promote foreign gene expression are mouse Ltk< - > and Chinese hamster ovary (CHO) dhfr< - > cells, although expression of Il-li is not limited to these cell lines.

3. Vector DNA should be introduced into these cell lines using any of a number of gene-transfer techniques The method employed here involves the calcium phosphate-DNA precipitation technique described by S. L. Graham & A. S. van der Eb (Virology 52:456-467, 1973) in which the expression vector for IL-li is co-precipitated with a second expression vector encoding a selectable marker. In the case of Ltk< - > cell transfection, the selectable marker is a thymidine kinase gene and the selection is as described by Wigler, et al. (Cell 16:777-785, 1979) and in the case of CHO dhfr< - > cells the selectable marker is dihydrofolate reductase (DHFR) whose selection is as described by Ringold et al. in J. Mol. Appl. Genet. 1:165-175, 1981.

4. Cells that express the IL-li gene constructs should then be grown under conditions that will increase the levels of production of IL-li. Cells carrying the metallothionein promoter constructs can now be grown in the presence of heavy metals such as cadmium which will lead to a 5-fold increased utilization of the MT-1 promoter (Mayo et al., Cell 29:99-108) subsequently leading to a comparable increase in IL-li protein levels. Cells containing IL-li expression vectors (either SV40- or MT-1-based) along with a DHFR expression vector can be taken through the gene amplification protocol described by Ringold et al. (J. Mol. Appl. Genet 1:165-175, 1981) using methotrexate, a competitive antagonist of DHFR. This leads to more copies of the DHFR genes present in the cells and, concomitantly, increased copies of the IL-li genes which, in turn, can lead to more IL-li protein being produced by the cells.
Example 9

Purification of Il-li From Recombinant Animal Cells

Since the IL-li are expected to be secreted from cells like the natural material, it is anticipated that the methods described above for purification of the natural protein will allow similar purification and characterization of the recombinant protein.
Example 10
Sequence of IL-li

The amino terminal residue of IL-li has been identified several times by direct protein sequencing as an arginine (R). The result of such sequencing is shown in Example 3. In contrast, the amino terminal residue of IL-li predicted by the sequence of the cDNA is a proline (P). This amino terminal residue corresponds to nucleotides 85-87 in FIG. 13, and is circled in FIGS. 14 and 15. This apparent disagreement between the cDNA sequence and the direct protein sequence can be resolved by assuming that an error in the cDNA sequence was incorporated during the reverse transcriptase-catalyzed synthesis from its mRNA. That is, a CGA (arginine) codon, located on the mRNA where it would code for that amino terminal residue, could have been changed during the reverse-transcriptase reaction to a CCA (proline) codon in the cDNA. This type of reverse transsscriptase problem has been reported in the literature before, e.g., by B. D. Clark et al. in Nucleic Acids Research 14:7897 (1986).

The present inventors believe that the correct amino acid sequence of the protein is as predicted by the cDNA except that the amino terminal amino acid is an arginine instead of the proline residue indicated in FIGS. 13-15. The inventors contemplate that both DNA sequences and their corresponding peptide sequences fall within the scope of their invention although the amino terminal arginine sequence is preferred.

CLAIMS: What is claimed is:

[*1] 1. An isolated DNA sequence encoding a physiologically functional interleukin-1 inhibitor (IL-li) comprising a DNA sequence that is selected from the group consisting of (1) a DNA sequence that encodes IL-li X, IL-li alpha, or IL-li beta and (2) a DNA sequence (i) that cross-hybridizes to a DNA sequence that encodes IL-li X, IL-li alpha, or IL-li beta or (ii) that cross-hybridizes to a DNA sequence that is complementary to a DNA sequence that encodes IL-li X, IL-li alpha, or IL-li beta, wherein said DNA sequence of (i) or (ii) encodes a protein having IL-1 inhibitor activity.

[*2] 2. The recombinant DNA molecular GT10-ILli-2A.

[*3] 3. The isolated DNA sequence of claim 1 wherein said DNA sequence comprises a DNA sequence that encodes IL-li X, IL-li alpha or IL-li beta.

[*4] 4. The isolated DNA sequence of claim 1, wherein said DNA base sequence includes the nucleic acids from position 99 to 554 from the sequence which follows: [See Original Patent for Chemical Structure Diagram]

[*5] 5. The isolated DNA sequence of claim 1, wherein said DNA base sequence includes the nucleic acids from position 99 to 554 from the sequence which follows: [See Original Patent for Chemical Structure Diagram]

[*6] 6. The recombinant DNA vector comprising the DNA sequence of claim 1.

[*7] 7. The vector of claim 6, wherein said vector is an expression vector and further

comprises at least one regulatory element needed for the expression of the DNA sequence in a host.

[*8] 8. The vector of claim 7, wherein said DNA sequence is capable of being expressed in bacteria.

[*9] 9. The vector of claim 7, wherein said DNA sequence is capable of being expressed in mammalian cells.

[*10] 10. The vector of claim 6 wherein said DNA sequence comprises a DNA sequence that encodes IL-li X, IL-li alpha or IL-li beta.

[*11] 11. A cell host including the vector of claim 6 inserted therein.

[*12] 12. The host cell of claim 11, wherein said host cell is capable of expressing said DNA sequence.

[*13] 13. The host cell of claim 12, wherein said host cell is a microorganism.

[*14] 14. The host cell of claim 13, wherein said host cell is a bacterial cell.

[*15] 15. The host cell of claim 14, wherein said host cell is Escherichia coli.

[*16] 16. The host cell of claim 13, wherein said host cell is a mammalian cell.

[*17] 17. A recombinant-DNA method for the production of an interleukin-1 inhibitor (IL-li) comprising:

(a) preparing a DNA sequence encoding a protein having IL-1 inhibitor activity, wherein said DNA sequence is selected from the group consisting of (1) a DNA sequence that encodes IL-li X, IL-li alpha, or IL-li beta and (2) a DNA sequence (i) that cross-hybridizes to a DNA sequence that encodes IL-li X, IL-li alpha, or IL-li beta or (ii) that cross-hybridizes to a DNA sequence that is complementary to a DNA sequence that encodes IL-li X, IL-li alpha, or IL-li beta, wherein said DNA sequence of (i) or (ii) encodes a protein having IL-1 inhibitor activity;

(b) subcloning the DNA sequence into a vector capable of being inserted into and replicated in a host cell, such vector containing at least one regulatory element needed to express the DNA sequence;

(c) inserting the vector containing the DNA sequence and at least one regulatory element into a host cell capable of expressing the DNA encoding the IL-1 inhibitor;

(d) culturing the host cell sunder conditions appropriate for replication of the vector and expression of the IL-1 inhibitor; and

(e) harvesting the IL-1 inhibitor.

[*18] 18. The method of claim 17 wherein said DNA sequence comprises a DNA sequence that encodes IL-li X, IL-li alpha or IL-li beta.

[*19] 19. The method of claim 17 wherein said DNA sequence is a cDNA.

[*20] 20. The method of claim 17 wherein said DNA sequence is a genomic sequence.

[*21] 21. The method of claim 17 wherein said DNA sequence is derived from mammalian

cells.

[*22] 22. The method of claim 21 wherein said DNA sequence is derived from human monocytes.

[*23] 23. The method of claim 17 wherein said host cell is a microorganism.

[*24] 24. The method of claim 23 wherein said microorganism is E. coli.

[*25] 25. The method of claim 17 wherein said host cells are mammalian cells.

[*26] 26. The method of claim 25 wherein said mammalian cells are CHO cells.

[*27] 27. The method of claim 17 wherein said DNA sequence is a synthetic polynucleotide.

[*28] 28. A recombinant-DNA method for the production of an interleukin-1 inhibitor (IL-li) comprising:

(a) culturing a host cell that includes inserted therein a vector comprising the DNA sequence of claim 1 operatively linked to at least one regulatory element needed for the expression of the DNA sequence in the host cell;

(b) harvesting the protein having IL-1 inhibitor activity.

[*29] 29. A recombinant-DNA method for the construction of an interleukin-1 inhibitor (IL-li) expression vector comprising:

(a) preparing the DNA sequence of claim 1; and

(b) subcloning the DNA sequence into a vector capable of being inserted into and replicated in a host cell, such vector containing at least one regulatory element needed for the expression of the DNA sequence.

[*30] 30. The method of claim 27 wherein said DNA sequence comprises a DNA sequence that encodes IL-li X, IL-li alpha or IL-li beta.

[*31] 31. The method of claim 28 wherein said DNA sequence comprises a DNA sequence that encodes IL-li X, IL-li alpha or IL-li beta.

Pat. No. 5,385,901, *

5,385,901

◆ GET 1st DRAWING SHEET OF 7

Jan. 31, 1995

Method of treating abnormal concentrations of TNF alpha

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INT-CL: [6] A61K 31#535; A61K 31#44; A61K 31#445

US-CL: 514#231.5; 514#231.2; 514#282; 514#327; 514#331; 514#348;

CL: 514;

SEARCH-FLD: 514#327, 348, 282, 231.2, 231.5, 331

REF-CITED:

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LEGAL-REP: Mathews, Woodbridge & Collins

CORE TERMS: alpha, compound, thalidomide, cell, concentration, monocyte, infection, protein, acid, formula, stimulated, effective, cytokine, secretion, patient, administered, cultured, mixture, assay, shock, inhibition, glutaminic, cachexia, treating, septic, blood, preparation, debilitating, therapeutic, supernatants

ABST:
Compounds of the structure [See Original Patent for Chemical Structure Diagram]

wherein R is selected from the group consisting of hydrogen, alkyl radicals of 1-6 carbon atoms, the phenyl radical, and the benzyl radical; and wherein R' is selected from the group consisting of the phthalimido radical and the succinimido radical and of the structure [See Original Patent for Chemical Structure Diagram] II

wherein X is CH2 or C=O; R" is H, -CH2CH3, -C6H5, -CH2C6H5, -CH2CH=CH2, or [See Original Patent for Chemical Structure Diagram]

and hydrolysis products of said compounds wherein R" is H and the piperidino ring or both the piperidino and the imido ring are hydrolyzed are useful for the control of abnormal

concentrations of TNF alpha manifested in septic shock, cachexia and HIV infection without substantially effecting the concentration of other cytokines.

NO-OF-CLAIMS: 18

EXMPL-CLAIM: 1

NO-OF-FIGURES: 12

NO-DRWNG-PP: 7

GOVT-INT:

This invention was made with Government support under AI 22616-05 AI 07012-25 awarded by the Allergy and Infections Institute. The Government has certain rights in the invention. RELATED APPLICATIONS

PARCASE:

This is a continuation of Ser. No. 07/834,588 filed Feb. 12, 1992, now abandoned, which in turn is a continuation of Ser. No. 07/655,087 filed Feb. 14, 1991, now abandoned.

SUM:

BACKGROUND OF THE INVENTION

Debilitation, i.e. loss of weight, strength, vascular weakness, and other symptoms are natural sequelae of many diseases which afflict humans. These may include, for example bacterial infections such as tuberculosis; viral infections, particularly retroviral infections including HIV infections such as AIDS; various forms of arthritis particularly rheumatoid and degenerative; ulcerative colitis; regional enteritis; and the like. Human patients with these symptoms may present with an acute condition such as septic shock or with a chronic condition such as cachexia.

U.S. Pat. No. 2,830,991 describes a class of therapeutic agents of the general formula I [See Original Patent for Chemical Structure Diagram] I

wherein R is selected from the group consisting of hydrogen, alkyl radicals containing 1-6 carbon atoms, the phenyl radical, and the benzyl radical; and wherein R' is selected from the group consisting of the phthalimido radical and the succinimido radical.

The subject matter of this patent and of any other patents or publications identified in this disclosure are incorporated herein by reference.

Preferred compounds within the scope of the above formula I, for use in this invention are:

3-phthalimido-2,6-dioxo-1-ethyl piperidine

3-phthalimido-2,6-dioxo-1-phenyl piperidine

3-phthalimido-2,6-dioxo-1-benzyl piperidine

3-phthalimido-2,6-dioxo-1-allyl piperidine

3-phthalimido-2,6-dioxo-piperidine

As described in the patent, the compounds are produced by reacting an aliphatic dicarboxylic acid, which contains five carbon atoms in a straight chain, the methylene groups of which are substituted by the substituents in accordance with the appropriate general formula, with urea or substitution products thereof or with a primary amine or an acid amide in such manner that water is split off and the ring is closed. If an amino group is present in the aliphatic chain, this group must not exist in free form in this stage of the process, since otherwise there is the danger of this amino group participating in an undesirable manner in the reaction. Instead of using the dicarboxylic acid, it is also possible to employ functional derivatives thereof, such as acid halides, acid esters and acid amides.

Compounds of the glutaminic acid series may be used as starting materials for the present invention. In this case also, the acid halides, esters and amides of glutaminic acid may be employed instead of the acid itself. It is known that glutaminic acids tends to form 5-membered rings with a free amino group. This reaction is undesirable for the purposes of the present invention. The amino group must therefore be substituted or protected prior to the ring-closing reaction. The protection of the amino group may be carried out, when using products of the glutaminic acid series, by introducing the phthalyl, succinyl or like radical in a manner known per se. The proportions of the components used for the ring formation must be such that at least 1 mol of the compound yielding the imide nitrogen is used to one mol of the glutaminic acid component.

The first compound listed above is prepared by reacting 27.7 g. of N-phthalyl glutaminic acid with 66 g. of a 33% solution of ethyl amine in water and slowly heating in an oil bath 160o-180o C., the mixture being maintained at this temperature for 15 to 20 minutes. The reaction product is recrystallised from alcohol by fractionation. It melts at 209o C.

The last compound listed above prepared by reacting 13 g. of phthalyl glutaminic acid anhydride and 6 g. of urea in 75 cc. of absolute xylene for 4 hours at the boiling point of the mixture. Formation of a sublimate takes place with evolution of ammonia and carbon dioxide. The xylene is then distilled off in vacuo and the residue recrystallized from 95% alcohol by fractionation. In addition to some phthalimide and phthalyl glutamine, the required N2-phthalyl glutaminic acid imide is obtained, having a melting point of 269o-271o C.

In the patent, the compounds are disclosed as having low toxicity and as useful for certain spasmolytic and antihistaminic effects. The compound 3-phthalimido-2,6-dioxopiperidine is disclosed as being particularly useful as a sedative. This compound was marketed as a sedative under the generic name thalidomide. It was subsequently discovered to be teratogenic and was withdrawn from the market.

Despite its teratogenicity, thalidomide has long been employed for the treatment of erythema nodosum leprosum (ENL) an accute inflammatory state occurring in lepromatous leprosy. See, for example Mellin, G. W., and M. Katzenstein. N. Engl. J. Med. 267:1184 (1962). More recently, it has been shown to be useful in the treatment of graft-versus-host disease by Vogelsany, G. B., S. Taylor, G. Gordon and A. D. Hess. Transplant Proc. 23:904 (1986); for treatment of reheumatoid arthritis by O. Gutierrez-Rodriguez, P. Starusta-Bacai and O. Gutierrez-Montes. The Journal of Rheumatology 16:2 158 (1989); and for treatment of aphthous ulceration in patients positive for HIV antibody. Brit. Med. J. 298:432 (1989).

The tumor necrosis factor (TNF- alpha) is one of several cytokines released mainly by mononulear phagocytes together with several other cytokines in response to stimuli to the immune system. It is required for a cell mediated immune response to overcome infections. As its name suggests, it is associated with the destruction of tumor cells. It is not present in

measureable amounts in normal sera, but appears, often very rapidly, in response to immunostimulators such as bacterial and viral infections, particularly HIV infections. In the case of chronic infection it may be found in the sera at relatively high or low levels for extended periods of time. It may also appear suddenly in high concentrations in response to release of a toxin by an invading bacteria. It is markedly elevated in ENL.

TNF- alpha has been recognized as manifesting a dose dependent toxicity. If present at low levels for too long a period it results in cachexia. At high levels even for a short time it results in septic shock.

Cachexia is a general weight loss and wasting occurring in the course of a chronic disease. More specifically, it is a weight loss not accounted for by decreased caloric intake. It is associated with cancer, the opportunistic infections of AIDS, inflammatory diseases, parasitic diseases, tuberculosis, high dose IL-2 therapy and the like. It is a chronic condition related to chronic diseases.

Septic shock is an acute condition usually, but not always attributed to infection or to toxic substances in the tissue. It is characterized by hypotension due to loss of vascular tone. It may result in patient collapse, or even death if not treated promptly and efficiently.

The retroviruses are a broad group of RNA viruses which, during their replication, employ the reverse transcription enzyme (RT) to convert a RNA message to DNA. The retroviridae family of viruses includes lentiviruses (visna, maedi, progressive pneumonia virus -"slow viruses"), spumaviruses (foamy viruses) and oncornaviruses (types A, B, C, D, RNA tumor viruses). The retroviruses have been shown to infect murine, avian, feline, primate, and human species.

The human immunodeficiency virus (HIV-1) or human T-Cell lymphotropic viruse (HTLV-III) which causes Acquired Immune Deficiency Syndrome (AIDS), AIDS related complex (ARC) and other AIDS related diseases is a retrovirus. TNF- alpha functions in an autocrine manner in the induction of HIV-1 expression (G. Poli et al, PNAs Vol 87 p 782, 1990).

It is apparent, therefore, that it is necessary to control the concentration of TNF- alpha in the sera to avoid the debilitating effects of abnormal concentrations of this cytokine including, for example, cachexia and septic shock.

Other cytokines which are necessary for a proper immune response are also produced by mononuclear phagocytes. These include, for example, various interleukins such as IL-1, IL-6, IL-8 and the granulocyte macrophage colony stimulating factor, GM-CSF. Still other cytokines are produced by the T-cells. It is desirable to control the concentration of TNF without appreciably affecting the concentration and activity of other cytokines.

Heretofore, antiinflammatory and immunosuppresive steroids such as prednisolone and dexamethasone have been employed to treat the debilitating effects of TNF- alpha . Unfortunately, these therapeutic agents also block the production of other cytokines so that the patients become susceptible to life threatening infections.
BRIEF SUMMARY OF THE INVENTION

It has now been discovered that the debilitating effects of toxic concentrations of TNF- alpha , whether acute or chronic, can be controlled in humans by treating a human patient in need of such treatment with an anti-debilitating amount of a compound within the scope of the above description. Typically the treatment may be either oral or parenteral, for example intravenously or subcutaneously.

It has further been discovered that certain compounds within the scope of the above formula as well as other closely related compounds are especially useful for the practice of this invention. These compounds are presently preferred for the therapeutic purposes of the

inventions. These preferred compounds include those represented by formula II [See Original Patent for Chemical Structure Diagram] II

wherein X is CH2 or C=O; R is H, -CH2CH3, -C6H5, -CH2C6H5, -CH2CH=CH2, or [See Original Patent for Chemical Structure Diagram]

and hydrolysis products of said compounds wherein R is H and the peperidino ring or both the peperidino and the imido ring are hydrolyzed.

Especially preferred compounds within the ambit of the above definition are represented by the formulas: [See Original Patent for Chemical Structure Diagram]

Most of the non-hydrolyzed compounds whose formulas are given above can be prepared by the processes described in the aforesaid U.S. Pat. No. 2,830,991. The preparation of the phthalimidine compounds is described in U.S. Pat. No. 3,705,162. U.S. Pat. No. 3,563,986 describes the preparation of the morpholino substituted compounds. The hydrolytic compounds are prepared by standard hydrolysis procedures several of which will be known to the skilled artisan.

The compounds used in the invention can exist as racemic mixtures. The racemic mixtures and separate isomers are included within the scope of the invention.

The compounds may be administered alone, but will normally be employed in a composition containing a pharmaceutically acceptable carrier. It may be advantageous, as will be discussed more fully below to administer the selected compound or compounds together with an effective amount of a therapeutic agent appropriate for treating the cause of the abnormal concentration of TNF- alpha , for example with an antibacterial agent if the condition under treatment is shock caused by the sudden release of large amounts of a toxin because of bacterial infection.

DRWDESC:
THE DRAWINGS

FIGS. 1a, 1b, 1c; 2, 3, show the effects of thalidomide on TNF- alpha production in the presence of various reagents.

FIGS. 4 through 7 show the results of studies conducted to establish the utility of the compounds of this invention to inhibit HIV-1 RT activity.

DETDESC:

The drawings and the balance of this disclosure will be better understood by recognizing the meanings of certain abbreviations. CWP-ML means cell wall protein of Mycobacterium leprae. ENL means erythema nodosum leprosum. GM-CSF means granulocyte macrophage colony-stimulating factor. PPD means purified protein derivative of tuberculin. PBMC means peripheral blood mononuclear cells.

The studies described hereinafter will be recognized by those skilled in the art as establishing that the compounds of this invention selectively inhibit the production of human TNF- alpha

without substantially affecting the production of other proteins or of total serum protein. Therefore, although the compounds of the invention will not cure diseases, they will significantly improve the quality of life of the patients. An important consequence of the study is the finding that TNF- alpha secretion is not totally inhibited. This is important since, as indicated above, TNF- alpha appears to be an essential mediator in the immune response.

There follows a complete description of one procedure for establishing the ability of the compounds of this invention to inhibit the production of TNF- alpha without inhibiting the production of other cytokines.
Monocyte Isolation.

PBMC obtained by Ficoll-Hypaque (Pharmacia Fine Chemicals, Piscataway, N.J.) density centrifugation were rosetted with neuraminidase-treated (Vibrio cholerae neuraminidase; Calbiochem-Behring Corp. La Jolla, Calif.) sheep erythrocytes (Scott Laboratories, Friskville, R.I.) (SRBC rosetting), and the nonrosetted cells were counted (E< - > population monocytes enriched). 10<6 > cells were cultured at 37o C. in 24-well plates (Corning Glass Works, Corning, N.Y.) in 1 ml of RPMI 1640 (Gibco Laboratories, Grand Island, N.Y.) supplemented with 10% AB< + > serum, 100 U/ml penicillin, 100 mu g/ml streptomycin, and 2 mM 1-glutamine. Adherent E< - > cells were used for the studies.
Cytokine Agonist

LPS of Salmonella minnesota R595 (List Biological Laboratories, Campbell, Calif.) was diluted in PBS, pH 7.4, and used at 1 g/ml; Purified protein derivative of tuberculin (PPD) was purchased from Statens Seruminstitut, Copenhagen, Denmark; CWP-ML was prepared using known and published methods. The concentrations of the stimulating agents were those known to induce optimal TNF- alpha protein production by cultured monocytes. The endotoxin content of solutions and mycobacterial preparations was estimated by the Limulus amebocyte lysate assay (LAL; Whittaker M. A. Bioproducts, Walkersville, Md.). All solutions used contained less than 10 pg/ml of endotoxin.
Cytokine Induction

Adherent E< - > cells were stimulated with 1 mu g/ml of LPS, 10 mu g/ml of PPD, or 10 mu g/ml of CWP-ML for up to 18-20 h. At various times, supernatants were harvested, centrifuged to remove cells and debris, and kept frozen until use (- 20o C.).
TNF- alpha Assay

TNF- alpha concentration in the supernatants was determined with a TNF- alpha specific ELISA, specific for the biologically active molecule. Assays were performed in 96-well plates (Nunc Immunoplates, Roskilde, Denmark) coated with the affinity-purified rabbit anti-TNF- alpha antibody (0.5 mu g/ml; 12-16 h; 4o C.) and blocked for 2 h at room temperature with PBS/0.05% Tween 20 (Sigma Chemical Co., St. Louis, Mo.) containing 5 mg/ml BSA. After washing, 100 mu l of TNF- alpha standards, samples, and controls were applied to the wells, and the plates were incubated for 12-24 h at 4o C. After the incubation, plates were washed and a second antibody, horseradish peroxidase (HRP)-conjugated mouse monoclonal anti- TNF- alpha , diluted 1:2,000 in PBS/BSA/Tween, was applied to the wells and incubated for 2 h at room temperature. The color reaction was developed with the OPD substrate (0.4 mg/ml o-phenylenediamine [Sigma Chemical Co.] in 24 mM citric acid, 51 mM sodium phosphate, pH 5.0 [phosphate-citrate buffer: Sigma Chemical Co.] containing 0.012% hydrogen peroxide [H2O2; Fisher Scientific Co., Pittsburgh, Pa.]) and absorbance read at 492 nm in an automated ELISA reader (Dynatech Laboratories, Inc., Alexandria, Va.).
IL-1 Assays

IL-1 levels were determined using a commercial ELISA kit (Cistron Biotechnology, Pine Brook, N.J.) according to the manufacturer's specifications. IL-1 levels are expressed as pico- grams per milliliter of protein.
IL-6 Assay

IL-6 levels were determined using a biological assay as described by Finkelman et al. Proc. Natl. Acad. Sci. USA. 83:9675 (1986). Proliferation of 7TD1 hybridoma cell line specifically sensitive to IL-6 was measured by colorimetric determination of hexosaminidase levels, Laudegren et al J. Immunol. Methods. 67:379 (1984), and values for IL-6 in the samples were obtained by interpolation from a standard curve. 1 U/ml of IL-6 corresponds to the concentration that yields half-maximal growth.
Granulocyte/Macrophage CSF GM-CSF Assay

GM-CSF levels were determined using a commercial ELISA kit (Genzyme, Boston, Mass.) according to the manufacturer's specifications, and were expressed as picograms per milliliter of protein.
Thalidomide Inhibition

The thalidomide used in this study was the purified drug (racemic mixture: D[+] and L [-] forms) (lot No. JB-I-114; Andrulis Research Corporation, Beltsville, Md.). The compound was shown to be at least 99% pure, as analyzed by Fourier Transform Infrared Spectrum. It was then diluted in DMSO (Sigma Chemical Co.); further dilutions were done in sterile PBS.

Percentage inhibition of TNF- alpha secretion was calculated as: 100 x [1-(TNF- alpha experimental/TNF- alpha control)]; where TNF- alpha experimental represents TNF-secretion by stimulated monocytes that were cultured in the presence of thalidomide, and TNF- alpha control represents TNF- alpha secretion by stimulated monocytes that were cultured in the absence of the drug. Monocytes cultured in medium containing equivalent amounts of DMSO in the presence or absence of the stimulating agent were used as controls for thalidomide-treated cells. Neither thalidomide nor DMSO had any effect on cell viability or function at the concentrations used.
Protein Synthesis

Human monocytes were cultured in Teflon beakers in methionine-free RPMI with 10% AB< + > serum at 37o C. for 1 h, when 200 mu Ci/ml<35> S-methionine (1,153 mu Ci/mmol; ICN Biomedicals Inc., Calif.) was added to the cultures for the next 3 h with or without the stimulating and the suppressive agent. At the end of the labeling period, <35> S-labeled cells were washed twice in ice-cold PBS and lysed directly in 500 mu l lysis solution (10 mM Tris-HCl buffer, pH 7.4 150 NaCl, 1 mM EDTA, and 1% SDS). Resolving 8% SDS-PAGE was performed overnight. The gel was washed, dried, and analyzed by autoradiography at - 70o C. using XAR-5 radiographic film (Kodak, Rochester, N.Y.) with an intensifying screen.
RESULTS OF THIS STUDY

Monocytes were enriched from PBMC of normal donors and stimulated in vitro for 18-20 h with bacterial LPS and mycobacterial products, known agonist of monocyte TNF- alpha synthesis and secretion. Thalidomide suppressed LPS-stimulated TNF- alpha production (FIG. 1A) with a 50% inhibitory concentration (IC50) of 1-4 mu g/ml, and 90% inhibition observed at 10 mu g/ml (18-20-h assay). Similar results were obtained when PPD and CWP-ML were used as stimulants (FIG. 1, B and C, respectively).

FIG. 1 shows the effect of thalidomide on (A) bacterial endotoxin (LPS, 1 mu g/ml), (B) PPD, (10 mu g/ml), and (C) CWP-ML (10 mu g/ml)- induced TNF- alpha production. Monocytes were stimultaneously incubated with 2 ng/ml to 10 mu g/ml of thalidomide in the culture medium. Control cells were cultrued in medium alone. A dose-dependent inhibition of TNF-alpha secretion by thalidomide is apparent. No detectable production of TNF- alpha protein was observed in supernatants of unstimulated monocytes. Data represent mean +/- SD of 15 (A), two (B), and one (C) different experiments, respectively.

The inhibition of TNF- alpha secretion by thalidomide was dependent upon the state of monocyte stimulation as shown in Table 1. Preincubation of unstimulated monocytes with

thalidomide, followed by removal of the drug before LPS stimulation, did not lead to suppression. By comparison, when LPS and thalidomide were added stimultaneously to the cultures, irreversible suppression occurred, even when the drug was removed after a few hours (Table 1). Therefore, the thalidomide sensitive reaction(s) occurs only after the LPS induction of TNF- alpha production.

TABLE 1

	h *			h			
A	0-4	0	0	4-20	0	+	100
B	0-4	+	0	4-20	0	+	90 +/- 4.6
C	None	0	0	0-4	+	+	48 +/- 15
D	0-4	+	+	4-20	0	+	56 +/- 0.5
E	None	0	0	0-20	+	+	52 +/- 9.3

Human monocytes cultured in 24-well plates were preincubated with the inhibitory drug with or without the stimulating agent. After 4 h, the cultures were washed, medium was replaced, and LPS was added again for the next 16 h. Culture supernatants were recovered at the different periods and TNF- alpha levels determined as described. LPS-inducted release of TNF- alpha by monocytes cultured for 20 h in the absence of thalidomide (A). No inhibitory action of thalidomide was detected when the drug was washed away before the addition of the stimulating agent (B). Thalidomide-induced inhibition of TNF- production in the present of LPS after 4 h of stimulation (C), which persisted even after the drug was washed away (D). Control experiment in which thalidomide was kept in the cultures with the stimulating agent during the whole assay (E). Data represent mean +/- SD of two different experiments.

The inhibition of LPS-stimulated TNF- alpha secretion by thalidomide occurs in a setting in which many other proteins are being synthesized by both constitutive and induced mechanisms. Thus, a simple explanation for the effect of the drug on TNF- alpha production could be a suppression of overall protein synthesis.

FIG. 2 illustrates the effect of thalidomide on the pattern and quantity of proteins synthesized after a 3-h pulse of <35> S-methionine. The total incorporation of isotope into TCA-precipitable proteins as well as the intensity of most of the individual bands on SDS-PAGE of LPS-triggered monocytes remained unchanged after thalidomide treatment.

In FIG. 2 can be seen the effect of thalidomide on protein synthesis by human peripheral blood monocytes. Electrophoretic analysis of lysates from monocytes incubated with <35> S-methionine was performed. Cells were stimulated in vitro with and without LPS in the presence or absence of thalidomide at 1 and 4 mu g/ml. TCA-precipitable radioactivity (10% TCA precipitation) was measured by liquid scintillation counting. The amount of radioactivity in the pellets expressed as cpm x 10< - 3 > and represents the mean of three precipitates with a SD of 10%. Neither total radioactivity nor the pattern of most of the protein bands in the gel was affected by thalidomide (lane 1) unstimulated cells, 3.3 x 10< - 2 > cpm in TCA precipitates; (lane 2) cells stimulated with 1 mu g/ml LPS, 4.2 x 10< - 2 > cpm in TCA precipitate; (lane 3) cells stimulated with LPS in the presence of 1 mu g/ml thalidomide, 4.2 x 10< - 2 > cmp in TCA precipitate; (lane 4) cells stimulated with LPS in the presence of mu g/ml thalidomide, 4.1 x 10< - 2 > cpm in TCA precipitate; (lanes 5 and 6) cells incubated only with thalidomide at 1 or 4 mu g/ml, respectively, 3.2 x 10< - 2 > and 2.8 x 10< - 2 > cpm in TCA precipitates, respectively.

Several cytokines are produced by monocytes in response to LPS in addition to TNF- alpha , including IL-1 and IL-6. FIG. 3 shows that thalidomide exerts a selective effect by

suppressing only TNF- alpha secretion LPS-stimulated monocytes. Whereas 4 mu g/ml thalidomide suppressed TNF- alpha production (41.9% inhibition) (FIG. 3A), neither IL-1 (FIG. 3B), IL-6 (FIG. 3 C), nor GM-CSF production (FIG. 3 D) was influenced by the drug. Similar but more extensive selective suppression was observed with much higher (up to 20 mu g/ml) concentrations of thalidomide. It was also observed that the D (+) enantiomer appeared to be more active than the L(-) enantiomer.

FIG. 3 shows the levels of different cytokines tested in culture supernatants of human monocytes stimulated with LPS for 6 h (A-C) or 20 h (D) in the presence or absence of 4 or 10 mu g/ml of thalidomide. Data represent mean +/- SD of six different experiments for TNF- alpha and IL-1 determinations and three experiments for IL-6 and GM-CSF measurements. About 41.9 +/- 14.6% and 52.8 +/- 14.7% inhibition of TNF- alpha secretion was found in the presence of 4 and 10 mu g/ml of thalidomide, respectively. "Cont" illustrates unstimulated cells cultured in medium. No effect on IL-1, IL-6, or GM-CSF secretion was detected in these cultures.

The following study establishes the utility of compounds of the invention for reducing TNF-alpha concentration in HIV infections. TNF- alpha is known to induce HIV replication. Similarly, it is known that peripheral blood monocytes from HIV infected patients secrete higher amounts of TNF- alpha than do monocytes from uninfected individuals. TNF- alpha is a cytokine capable of inducing viral expression in cells chronically infected with HIV. The art, therefore, has long been concerned with discovering products capable of inhibiting TNF-alpha production in HIV infected patients. The compounds of this invention are capable of so doing. This fact was established in studies using the known and commercially available chronically infected cell lines U1 and ACH-2, a promonocytic cell line and a T-lymphocytic cell line. The procedure employed is described by Poli et al. (1990) Proc. Nat'l. Acad. sci. U.S.A. Vol. 87, pp 782-785.

Briefly, the expression of HIV was upregulated by the addition of 10< - 7 > m of phorbol 12-myristate 13-acetate (PMA) or 1 mu g/ml of TNF alpha to ACH-2 and U1 cells. The cells were suspended at 4 x 10<5 > per ml in RMP1 1640 medium (M.A. Bioproducts) supplemented with 10% (vol/vol) fetal calf serum in the presence of the selected amount of stimulator at 37o C. in 5% CO2/95% air for 48 hours, the supernatants collected and tested for the presence of Mg< + + > dependent reverse transcriptase activity using the procedure of Willy et al (1988) J. Virol. 62, 139-147.

For the test, 10 mu l of supernatants were added to 50 mu l of a mixture containing 5 mu g per ml of poly(rA) p(dT) 12-18, (Pharmacia), 5 mM MgCl2 and 10 mu ci/ mu l of <32> P-labeled deoxythymidine 5'- triphosphate (dTTP-Amersham), and the mixture was incubated for 1 1/2 hours at 37o C. Eight microliters of the mixture were spotted onto DE81 paper (Whatman), air-dried and washed 5 times in 2 x standard saline citrate buffer, and two additional times with 95% ethanol. The paper was dried, cut and radioactivity assayed. The results are shown in the figures.

FIG. 4 shows the results of tests in which 5, 10 and 50 mu g/ml of thalidomide (THAL) and the known TNF alpha inhibitor pentoxyfylline (PTN) were used to inhibit reverse transcriptase production with the cell line U1. For the comparison, reverse transcriptase activity in the absence of the inhibitor was taken as 100%. It will be seen that at a concentration of 50 mu g/ml, thalidomide was as effective as PTN.

FIG. 5 shows the results of a similar test with a U1 cell line stimulated with PMA comparing thalidomide and PTN with other compounds of the invention including the D isomer of thalidomide. The other compounds of the invention are identified in this and the following figures by the letters used under their formulas hereinabove.

FIG. 6 shows a similar study in which the same compounds were tested with ACH-2

stimulated with TNF- alpha .

FIG. 7 records the results of a test using the ACH-2 cell line stimulated with PMA.

The compounds of the invention or their pharmaceutically acceptable salts may be administered perorally in a pharmaceutical carrier in standard form such as tablets, pills, lozenges, dragees and similar shaped and/or compressed preparations. It is also possible to produce emulsions or suspensions of the compounds in water or aqueous media such as unsweetened fruit juices and by means of suitable emulsifying or dispersing agents. They may also be employed in the form of powders filled into gelatin capsules or the like.

Such powders and mixtures for use in the preparation of tablets and other shaped and/or compressed preparations may be diluted by mixing and milling with a solid pulverulent extending agent to the desired degree or firmness or by impregnating the already milled, finely powdered, solid carrier with a suspension of the compounds in water or with a solution thereof in an organic solvent and then removing the water or solvent.

When preparing tablets, pills, dragees, and the like shaped and/or compressed preparations, the commonly used diluting, binding, and disintegrating agents, lubricants, and other tableting adjuvants are employed, provided they are compatible with agent to be administered. Such diluting agents and other excipients are, for instance, sugar, lactose, levulose, starch, bolus alba; as disintegrating and binding agents, gelatin, gum arabic, yeast extract, agar, tragacanth, methyl cellulose, pectin: and as lubricants stearic acid, talc, magnesium stearate, and others.

They may be administered in the form of suppositories, typically utilizing such commonly used suppository vehicles, as cocoa butter.

The compounds may also be administered parenterally employing aqueous solutions or suspensions of watersoluble compounds or suspensions. The compositions may be made isotonic e.g. with salt or other solute and may contain a buffer, for example a phosphate buffer.

As indicated above, the compound employed in the invention may be the only active ingredient administered or it may be coadministered with another therapeutic agent in an amount which is effective to treat the condition associated with the debilitating effect. For example, if the cause of the condition is a toxin released by an infectious bacteria, an antibiotic such as tetracycline, penicillin, streptomycin and the like may be coadministered. If there is hypotension associated with lack of vascular tone, a vasopressive agent such as epinephrine or dopamine may be coadministered. If the patient is under treatment with a chemotherapeutic agent such as adriamycin, the compound of the invention and the chemotherapeutic agent may be coadministered.

The term "coadministered" does not mean that the compound of the invention and the additional therapeutic agent are administered in the same dosage unit, although they may be so administered. It means that they are administered within the same time span.

An "effective amount" of the compound or additional therapeutic agent will vary with the condition being treated, the age, weight and general physical condition of the patient under treatment and other factors readily evaluated by the physician in attendance.

CLAIMS: What is claimed is:

[*1] 1. The method of treating the toxic symptoms of high concentrations of TNF alpha manifested in septic shock, cachexia, and HIV infection by inhibiting the production of TNF alpha which comprises administering to a human susceptible to or exhibiting such symptoms

an effective amount of a compound of the formula: [See Original Patent for Chemical Structure Diagram]

in which R is hydrogen, alkyl of 1 to 6 carbon atoms, phenyl, or benzyl, and

R' is [See Original Patent for Chemical Structure Diagram]

[*2] 2. The method of claim 1 wherein R' is [See Original Patent for Chemical Structure Diagram]

[*3] 3. The method of claim 2 wherein said compound is 3-phthalimido-2,6-dioxopiperidine.

[*4] 4. The method of claim 2 wherein said effective amount is sufficient to produce a blood level of said compound of at least 0.1 mu g/mL.

[*5] 5. The method of treating the debilitating effects of septic shock caused by high concentrations of TNF alpha by inhibiting production of TNF alpha which comprises administering to a human susceptible to or exhibiting such effects an effective amount of a compound of the formula: [See Original Patent for Chemical Structure Diagram]

in which R is hydrogen, alkyl of 1 to 6 carbon atoms, phenyl, or benzyl, and

R' is [See Original Patent for Chemical Structure Diagram] or [See Original Patent for Chemical Structure Diagram]

[*6] 6. The method of claim 5 wherein R' is [See Original Patent for Chemical Structure Diagram]

[*7] 7. The method of claim 6 wherein said compound is 3-phthalimido-2,6-dioxopiperidine.

[*8] 8. The method of claim 6 wherein said effective amount is sufficient to produce a blood level of said compound of at least 0.1 mu g/mL.

[*9] 9. The method of treating the debilitating effects of cachexia caused by high concentrations of TNF alpha by inhibiting production of TNF alpha which comprises administering to a human susceptible to or exhibiting such effects an amount of a compound of the formula: [See Original Patent for Chemical Structure Diagram]

in which R is hydrogen, alkyl of 1 to 6 carbon atoms, phenyl, or benzyl, and

R' is [See Original Patent for Chemical Structure Diagram] or [See Original Patent for Chemical Structure Diagram]

[*10] 10. The method of claim 9 wherein R' is [See Original Patent for Chemical Structure Diagram]

[*11] 11. The method of claim 10 wherein said compound is 3-phthalimido-2,6-dioxopiperidine.

[*12] 12. The method of claim 10 wherein said effective amount is sufficient to produce a blood level of said compound of at least 0.1 mu g/mL.

[*13] 13. The method of treating the debilitating effects of an HIV infection caused by high concentrations of TNF alpha by inhibiting production of TNF alpha which comprises administering to a human susceptible to or exhibiting such effects an amount of a compound of the formula: [See Original Patent for Chemical Structure Diagram]

in which R is hydrogen, alkyl of 1 to 6 carbon atoms, phenyl, or benzyl, and

R' is [See Original Patent for Chemical Structure Diagram] or [See Original Patent for Chemical Structure Diagram]

[*14] 14. The method of claim 13 wherein R' is [See Original Patent for Chemical Structure Diagram]

[*15] 15. The method of claim 14 wherein said compound is 3-phthalimido-2,6-dioxopiperidine.

[*16] 16. The method of claim 14 wherein said effective amount is sufficient to produce a blood level of said compound of at least 0.1 mu g/mL.

[*17] 17. The method of treating the toxic symptoms of high concentrations of TNF alpha manifested in septic shock, cachexia, and HIV infection by inhibiting the production of TNF alpha which comprises administering to a human susceptible to or exhibiting such symptoms an effective amount of a compound of the formula: [See Original Patent for Chemical Structure Diagram]

in which R is allyl or morpholinomethyl, and

R' is [See Original Patent for Chemical Structure Diagram] or [See Original Patent for Chemical Structure Diagram]

[*18] 18. The method of claim 17 wherein said effective amount is sufficient to produce a blood level of at least 0.1 mu g/mL.

Source: All Sources : / . . . / : **Utility, Design and Plant Patents** ⓘ
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♦ GET 1st DRAWING SHEET OF 36

Aug. 12, 1997

Methods of treating TNF- alpha -mediated Crohn's disease using chimeric anti-TNF antibodies

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Feb. 2, 1993 now abandoned Which is a continuation-in-part of Ser. No. 943,852, Sep. 11,
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LEGAL-REP: Hamilton, Brook, Smith & Reynolds, P.C.

CORE TERMS: region, anti-tnf, antibody, sequence, alpha, patient, fragment, chimeric, protein, peptide, gene, ca2, binding, murine, mab, fusion, molecule, receptor, disease, heavy, neutralizing, site, amino acids, epitope, assay, vector, immunoglobulin, residue, amino acid, constant

ABST:
Anti-TNF antibodies, fragments and regions thereof which are specific for human tumor necrosis factor- alpha (TNF alpha) and are useful in vivo for diagnosis and therapy of a number of TNF alpha -mediated pathologies and conditions, including Crohn's disease, as well as polynucleotides coding for murine and chimeric antibodies, methods of producing the antibody, methods of use of the anti-TNF antibody, or fragment, region or derivative thereof, in immunoassays and immunotherapeutic approaches are provided.

NO-OF-CLAIMS: 7

EXMPL-CLAIM: 1

NO-OF-FIGURES: 48

NO-DRWNG-PP: 36

PARCASE: This application is a continuation-in-part of each of U.S. application Ser. No. 08/010,406, filed Jan. 29, 1993, now abandoned, and U.S. application Ser. No. 08/013,413, filed Feb. 2, 1993, now abandoned, which is a continuation-in-part of U.S. application Ser. No. 07/943,852, filed Sep. 11, 1992, now abandoned, which is a continuation-in-part of U.S. application Ser. No. 07/853,606, filed Mar. 18, 1992, now abandoned, which is a continuation-in-part of U.S. application Ser. No. 07/670,827, filed Mar. 18, 1991, now abandoned. Each of the above non-abandoned applications is entirely incorporated herein by

reference.

SUM:

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention in the field of immunology and medicine relates to anti-tumor necrosis factor (TNF) antibodies, anti-TNF peptides and nucleic acids encoding therefor, and to pharmaceutical and diagnostic compositions and production, diagnostic and therapeutic methods thereof, and to methods for treating human TNF-mediated pathologies.

2. Description of the Background Art

Tumor Necrosis Factor: Monocytes and macrophages secrete cytokines known as tumor necrosis factor- alpha (TNF alpha) and tumor necrosis factor- beta (TNF beta) in response to endotoxin or other stimuli. TNF alpha is a soluble homotrimer of 17 kD protein subunits (Smith, et al., J. Biol. Chem. 262: 6951-6954 (1987)). A membrane-bound 26 kD precursor form of TNF also exists (Kriegler, et al., Cell 53: 45-53 (1988)). For reviews of TNF, see Beutler, et al., Nature 320: 584 (1986), Old, Science 230: 630 (1986), and Le, et al., Lab. Invest. 56: 234

Cells other than monocytes or macrophages also make TNF alpha . For example, human non-monocytic tumor cell lines produce TNF (Rubin, et al., J. Exp. Med. 164: 1350 (1986); Spriggs, et al., Proc. Natl. Acad. Sci. USA 84: 6563 (1987)). CD4< + > and CD8< + > peripheral blood T lymphocytes and some cultured T and B cell lines (Cuturi, et al., J. Exp. Med. 165: 1581 (1987); Sung, et al., J. Exp. Med. 168: 1539 (1988)) also produce TNF alpha .

TNF causes pro-inflammatory actions which result in tissue injury, such as inducing procoagulant activity on vascular endothelial cells (Pober, et al., J. Immunol. 136: 1680 (1986)), increasing the adherence of neutrophils and lymphocytes (Pober, et al., J. Immunol. 138: 3319 (1987)), and stimulating the release of platelet activating factor from macrophages, neutrophils and vascular endothelial cells (Camussi, et al., J. Exp. Med. 166: 1390 (1987)).

Recent evidence associates TNF with infections (Cerami, et al., Immunol. Today 9: 28 (1988)), immune disorders, neoplastic pathologies (Oliff, et al., Cell 50: 555 (1987)), autoimmune pathologies and graft-versus host pathologies (Piguet, et al., J. Exp. Meg. 166: 1280 (1987)). The association of TNF with cancer and infectious pathologies is often related to the host's catabolic state. Cancer patients suffer from weight loss, usually associated with anorexia.

The extensive wasting which is associated with cancer, and other diseases, is known as "cachexia" (Kern, et al. (J. Parent. Enter. Nutr. 12: 286-298 (1988)). Cachexia includes progressive weight loss, anorexia, and persistent erosion of body mass in response to a malignant growth. The fundamental physiological derangement can relate to a decline in food intake relative to energy expenditure. The cachectic state causes most cancer morbidity and mortality. TNF can mediate cachexia in cancer, infectious pathology, and other catabolic states.

TNF also plays a central role in gram-negative sepsis and endotoxic shock (Michie, et al., Br. J. Surg. 76: 670-671 (1989); Debets, et al., Second Vienna Shock Forum, p. 463-466 (1989); Simpson, et al., Crit. Care Clin. 5: 27-47 (1989)), including fever, malaise, anorexia, and cachexia. Endotoxin strongly activates monocyte/macrophage production and secretion

of TNF and other cytokines (Korribluth, et al., J. Immunol. 137: 2585-2591 (1986)). TNF and other monocyte-derived cytokines mediate the metabolic and neurohormonal responses to endotoxin (Michie, et al., New. Engl. J. Med. 318: 1481-1486 (1988)). Endotoxin administration to human volunteers produces acute illness with flu-like symptoms including fever, tachycardia, increased metabolic rate and stress hormone release (Revhaug, et al., Arch. Surg. 123: 162-170 (1988)). Circulating TNF increases in patients suffering from Gram-negative sepsis (Waage, et al., Lancet 1: 355-357 (1987); Hammerle, et al., Second Vienna Shock Forum p. 715-718 (1989); Debets, et al., Crit. Care Med. 17: 489-497 (1989); Calandra, et al., J. Infect. Dis. 161: 982-987 (1990)).

TNF Antibodies

Polyclonal murine antibodies to TNF are disclosed by Cerami et al. (EPO Patent Publication 0212489, Mar. 4, 1987). Such antibodies were said to be useful in diagnostic immunoassays and in therapy of shock in bacterial infections.

Rubin et al. (EPO Patent Publication 0218868, Apr. 22, 1987) discloses murine monoclonal antibodies to human TNF, the hybridomas secreting such antibodies, methods of producing such murine antibodies, and the use of such murine antibodies in immunoassay of TNF.

Yone et al. (EPO Patent Publication 0288088, Oct. 26, 1988) discloses anti-TNF murine antibodies, including mAbs, and their utility in immunoassay diagnosis of pathologies, in particular Kawasaki's pathology and bacterial infection. The body fluids of patients with Kawasaki's pathology (infantile acute febrile mucocutaneous lymph node syndrome; Kawasaki, Allergy 16: 178 (1967); Kawasaki, Shonica (Pediatrics) 26: 935 (1985)) were said to contain elevated TNF levels which were related to progress of the pathology (Yone et al., infra).

Other investigators have described rodent or murine mAbs specific for recombinant human TNF which had neutralizing activity in vitro (Liang, et al. (Biochem. Biophys. Res. Comm. 137: 847-854 (1986); Meager, et al., Hybridoma 6: 305-311 (1987); Fendly et al., Hybridoma 6: 359-369 (1987); Bringman, et al., Hybridoma 6: 489-507 (1987); Hirai, et al., J. Immunol. Meth. 96: 57-62 (1987); Moller, et al. (Cytokine 2: 162-169 (1990)). Some of these mAbs were used to map epitopes of human TNF and develop enzyme immunoassays (Fendly et al., infra; Hirai et al., infra; Moller et al., infra) and to assist in the purification of recombinant TNF (Bringman et al., infra). However, these studies do not provide a basis for producing TNF neutralizing antibodies that can be used for in vivo diagnostic or therapeutic uses in humans, due to immunogenicity, lack of specificity and/or pharmaceutical suitability.

Neutralizing antisera or mAbs to TNF have been shown in mammals other than man to abrogate adverse physiological changes and prevent death after lethal challenge in experimental endotoxemia and bacteremia. This effect has been demonstrated, e.g., in rodent lethality assays and in primate pathology model systems (Mathison, et al., J. Clin. Invest. 81: 1925-1937 (1988); Beutler, et al., Science 229: 869-871 (1985); Tracey, et al., Nature 330: 662-664 (1987); Shimamoto, et al., Immunol. Lett. 17: 311-318 (1988); Silva, et al., J. Infect. Dis. 162: 421-427 (1990); Opal, et al., J. Infect. Dis. 161: 1148-1152 (1990); Hinshaw, et al., Circ. Shock 30: 279-292 (1990)).

Putative receptor binding loci of hTNF has been disclosed by Eck and Sprang (J. Biol. Chem. 264 (29), 17595-17605 (1989), who identified the receptor binding loci of TNF- alpha as consisting of amino acids 11-13, 37-42, 49-57 and 155-157.

PCT publication WO91/02078 (1991) discloses TNF ligands which can bind to monoclonal antibodies having the following epitopes: at least one of 1-20, 56-77, and 108-127; at least two of 1-20, 56-77, 108-127 and 138-149; all of 1-18, 58-65, 115-125 and 138-149; all of 1-18, and 108-128; all of 56-79, 110-127 and 135- or 136-155; all of 1-30, 117-128 and

141-153; all of 1-26, 117-128 and 141-153; all of 22-40, 49-96 or 49-97, 110-127 and 136-153; all of 12-22, 36-45, 96-105 and 132-157; both of 1-20 and 76-90; all of 22-40, 69-97, 105-128 and 135-155; all of 22-31 and 146-157; all of 22-40 and 49-98; at least one of 22-40, 49-98 and 69-97, both of 22-40 and 70-87.

To date, experience with anti-TNF murine mAb therapy in humans has been limited. In a phase I study, fourteen patients with severe septic shock were administered a murine anti-TNF mAb in a single dose from 0.4-10 mg/kg (Exley, A. R. et al., Lancet 335: 1275-1277 (1990)). However, seven of the fourteen patients developed a human anti-murine antibody response to the treatment, which treatment suffers from the known problems due to immunogenicity from the use of murine heavy and light chain portions of the antibody. Such immunogenicity causes decreased effectiveness of continued administration and can render treatment ineffective, in patients undergoing diagnostic or therapeutic administration of murine anti-TNF antibodies.

Administration of murine TNF mAb to patients suffering from severe graft versus host pathology has also been reported (Herve, et al., Lymphoma Res. 9: 591 (1990)).

TNF Receptors

The numerous biological effects of TNF alpha and the closely related cytokine, TNF beta (lymphotoxin), are mediated by two TNF transmembrane receptors, both of which have been cloned. The p55 receptor (also termed TNF-R55, TNF-RI, or TNFR beta) is a 55 kd glycoprotein shown to transduce signals resulting in cytotoxic, anti-viral, and proliferative activities of TNF alpha .

The p75 receptor (also termed TNF-R75, TNF-RII, or TNFR alpha) is a 75 kDa glycoprotein that has also been shown to transduce cytotoxic and proliferative signals as well as signals resulting in the secretion of GM-CSF. The extracellular domains of the two receptors have 28% homology and have in common a set of four subdomains defined by numerous conserved cysteine residues. The p75 receptor differs, however, by having a region adjacent to the transmembrane domain that is rich in proline residues and contains sites for O-linked glycosylation. Interestingly, the cytoplasmic domains of the two receptors share no apparent homology which is consistent with observations that they can transduce different signals to the interior of the cell.

TNF alpha inhibiting proteins have been detected in normal human urine and in serum of patients with cancer or endotoxemia. These have since been shown to be the extra-cellular domains of TNF receptors derived by proteolytic cleavage of the transmembrane forms. Many of the same stimuli that result in TNF alpha release also result in the release of the soluble receptors, suggesting that these soluble TNF alpha inhibitors can serve as part of a negative feedback mechanism to control TNF alpha activity.

Aderka, et al., Isrl. J. Med. Sci. 28: 126-130 (1992) discloses soluble forms of TNF receptors (sTNF-Rs) which specifically bind TNF and thus can compete with cell surface TNF receptors to bind TNF (Seckinger, et al., J. Exp. Med. 167: 1511-1516 (1988); Engelmann, et al., J. Biol. Chem. 264: 11974-11980 (1989)).

Loetscher, et al., Cell 61: 351-359 (Apr. 20, 1990) discloses the cloning and expression of human 55 kd TNF receptor with the partial amino acid sequence, complete cDNA sequence and predicted amino acid sequence.

Schall et al., Cell 61: 361-370 (Apr. 20, 1990), discloses molecular cloning and expression of a receptor for human TNF wherein an isolated cDNA clone including a receptor as a 415 amino acid protein with an apparent molecular weight of 28 kDa, as well as the cDNA sequence and predicted amino acid sequence.

Nophar, et al., EMBO J. 9 (10): 3269-3278 (1990) discloses soluble forms of TNF receptor and that the cDNA for type I TNF-R encodes both the cell surface and soluble forms of the receptor. The cDNA and predicted amino acid sequences are disclosed.

Engelmann, et al., J. Biol. Chem. 265 (3): 1531-1536 (1990), discloses TNF-binding proteins, purified from human urine, both having an approximate molecular weight of 30 kDa and binding TNF- alpha more effectively than TNF- beta . Sequence data is not disclosed. See also Engelmann, et al., J. Biol. Chem. 264 (20): 11974-11980 (1989).

European Patent publication number 0 433 900 A1, published Jun. 26, 1991, owned by YEDA Research and Developmetn Co., Ltd., Wallach, et al., discloses TNF binding protein I (TBP-I), derivatives and analogs thereof, produced expression of a DNA encoding the entire human type I TNF receptor, or a soluble domain thereof.

PCT publication number WO 92/13095, published Aug. 6, 1992, owned by Synergen, Carmichael et al., discloses methods for treating tumor necrosis factor mediated diseases by administration of a therapeutically effective amount of a TNF inhibitor selected from a 30 kDa TNF inhibitor and a 40 kDa TNF inhibitor selected from the full length 40 kDa TNF inhibitor or modifications thereof.

European Patent Publication number 0 526 905 A2, published Oct. 2, 1993, owned by YEDA Research one Development Company, Ltd., Wallach et al., discloses multimers of the soluble forms of TNF receptors produced by either chemical or recombinant methods which are useful for protecting mammals from the diliterious effects of TNF, which include portions of the hp55 TNF-receptor.

PCT publication WO 92/07076, published Apr. 30, 1992, owned by Charring Cross Sunley Research Center, Feldman et al., discloses modified human TNF alpha receptor which consists of the first three cysteine-rich subdomain but lacks the fourth Cysteine-rich subdomain of the extracellular binding domain of the 55 kDa or 75 kDa TNF receptor for human TNF alpha , or an amino acid sequence having a homology of 90% or more with the TNF receptor sequences.

European Patent Publication 0 412 486 A1, published Feb. 13, 1991, owned by YEDA Research and Development Co., Ltd., Wallach et al., discloses antibodies to TNF binding protein I (TBP-I), and fragments thereof, which can be used as diagnostic assays or pharmaceutical agents, either inhibiting or mimicking the effects of TNF on cells.

European Patent Publication number 0 398 327 A1, published Nov. 22, 1990, owned by YEDA Research and Development Co., Ltd., Wallach et al., discloses TNF binding protein (TBP) isolated and purified having inhibitory activity on the cytotoxic effect of TNF, as well as TNF binding protein II and salts, functional derivatives precursors and active fractions thereof, as well as polyclonal and monoclonal antibodies to TNF binding protein II.

European Patent Publication 0 308 378 A2, published Mar. 22, 1989, owned by YEDA Research and Developmetn Co., Ltd., Wallach, et al., discloses TNF inhibitory protein isolated and substantially purified, having activity to inhibit the binding of TNF to TNF receptors and to inhibit the cytotoxicity of TNF. Additionally disclosed are TNF inhibitory protein, salts, functional derivatives and active fractions thereof, used to antagonize the diliterious effects of TNF.

Accordingly, there is a need to provide novel TNF antibodies or peptides which overcome the problems of murine antibody immunogenicity and which provide reduced immunogenicity and increased neutralization activity.

Citation of documents herein is not intended as an admission that any of the documents cited herein is pertinent prior art, or an admission that the cited documents is considered material to the patentability of any of the claims of the present application. All statements as to the date or representation as to the contents of these documents is based on the information available to the applicant and does not constitute any admission as to the correctness of the dates or contents of these documents.

SUMMARY OF THE INVENTION

It is object of the present invention to overcome one or more deficiencies of the background art.

It is also an object of the present invention to provide methods having utility for in vitro, in situ and/or in vivo diagnosis and/or treatment of animal cells, tissues or pathologies associated with the presence of tumor necrosis factor (TNF), using anti-TNF antibodies and/or anti-TNF peptides.

Anti-TNF antibodies (Abs) are intended to include at least one of monoclonal rodent-human chimeric antibodies, rodent antibodies, human antibodies or any portions thereof, having at least one antigen binding region of an immunoglobulin variable region, which antibody binds TNF.

Anti-TNF peptides are capable of binding TNF under physiological conditions, and can include, but are not limited to, portions of a TNF receptor and/or portions or structural analogs of anti-TNF antibody antigen binding regions or variable regions. Such antibodies or peptides bind TNF with neutralizing and/or inhibiting biological activity.

Anti-TNF antibodies and/or anti-TNF peptides of the present invention can be routinely made and/or used according to methods of the present invention, such as, but not limited to synthetic methods, hybridomas, and/or recombinant cells expressing nucleic acid encoding such anti-TNF antibodies or peptides.

The present invention also provides antigenic polypeptides of hTNF, corresponding to peptides containing neutralizing epitopes or portions of TNF that, when such epitopes on TNF are bound by anti-TNF antibodies or peptides, neutralize or inhibit the biological activity of TNF in vitro, in situ or in vivo.

The present invention also provides anti-TNF antibodies and peptides in the form of pharmaceutical and/or diagnostic compounds and/or compositions, useful for the diagnostic and/or therapeutic methods of the present invention for diagnosing and/or treating TNF-related pathologies.

Anti-TNF Abs or anti-TNF peptides of the present invention are provided for use in diagnostic methods for detecting TNF in patients or animals suspected of suffering from conditions associated with abnormal TNF production, including methods wherein high affinity anti-TNF antibodies or peptides are contacted with a biological sample from a patient and an antigen-antibody reaction detected. Also included in the present invention are kits for detecting TNF in a solution using anti-TNF antibodies or peptides, preferably in detectably labeled form.

The present invention is also directed to an anti-hTNF chimeric antibody comprising two light chains and two heavy chains, each of the chains comprising at least part of a human constant region and at least part of a variable (V) region of non-human origin having specificity to human TNF, said antibody binding with high affinity to a inhibiting and/or neutralizing epitope of human TNF, such as the antibody cA2. The invention is also includes a fragments or a derivative such an antibody, such as one or more portions of the antibody chain, such as the

heavy chain constant, joining, diversity or variable regions, or the light chain constant, joining or variable regions.

Methods are also provided for making and using anti-TNF antibodies and peptides for various utilities of the present invention, such as but not limited to, hybridoma, recombinant or chemical synthetic methods for producing anti-TNF antibodies or anti-TNF peptides according to the present invention; detecting TNF in a solution or cell; removing TNF from a solution or cell, inhibiting one or more biological activities of TNF in vitro, in situ or in vitro. Such removal can include treatment methods of the present invention for alleviating symptoms or pathologies involving TNF, such as, by not limited to bacterial, viral or parasitic infections, chronic inflammatory diseases, autoimmune diseases, malignancies, and/or neurodegenerative diseases.

DRWDESC:
BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a graph showing dose dependent binding of mouse mAb A2 to human TNF alpha .

FIG. 2 is a graph showing lack of recognition of heat-inactivated human TNF alpha by mAb A2.

FIG. 3 is a graph showing neutralization of in vitro TNF cytotoxicity by murine A2. Control: murine IgG1 anti-lipid A mAb (8A1) with natural human TNF. Average absorbance values for controls were as follows: no TNF added = 1.08; natural TNF, no antibody = 0.290; and recombinant TNF, no antibody = 0.500.

FIG. 4 is a graph showing that mAb A2 and chimeric A2 do not inhibit or neutralize human lymphotoxin (TNF beta).

FIG. 5 is a graph showing that mAbs murine A2 and chimeric CA2 do not inhibit or neutralize murine TNF alpha .

FIG. 6 and FIG. 7 are graphs showing that mAb A2 inhibits or neutralizes TNF produced by chimpanzee monocytes and rhTNF alpha .

FIGS. 8A-8B provide schematic diagrams of the plasmids used for expression of the chimeric H (pA2HG1apgpt) and L (pA2HuKapgpt) chains of the chimeric A2 antibody.

FIGS. 9A-9B provide graphs showing results of a cross-blocking epitope ELISA with murine A2 (mA2) and chimeric (cA2) antibody competitors.

FIGS. 10A-B provide graphs graph of a Scatchard analysis of <125> I-labelled mAb A2 (mA2) and chimeric A2 (cA2) binding to recombinant human TNF alpha immobilized on a microtiter plate. Each Ka value was calculated from the average of two independent determinations.

FIG. 11 is a graph showing neutralization of TNF cytotoxicity by chimeric A2. The control is a chimeric mouse/human IgG1 anti-platelet mAb (7E3) reacting with natural human TNF. Average absorbance values for controls were: no TNF added = 1.08; natural TNF, no Ab = 0.290; and recombinant TNF, no Ab = 0.500.

FIG. 12 is a graph showing in vitro neutralization of TNF-induced ELAM-1 expression by chimeric A2. The control is a chimeric mouse/human IgG1 anti-CD4 antibody.

FIG. 13 is an amino acid sequence of human TNF as SEQ ID NO:1.

FIG. 14A is a graphical representation of epitope mapping of chimeric mAb cA2 indicating relative binding of cA2 to human TNF peptide pins.

FIG. 14B is a graphical representation of epitope mapping of chimeric mAb cA2 indicating relative binding of cA2 to human TNF peptide pins in the presence of human TNF.

FIG. 15 is an amino acid sequence of human TNF showing sequences having portions of epitopes recognized by cA2, corresponding to portions of amino acids 59-80 and/or 87-108 of SEQ ID NO:1.

FIG. 16A is a nucleic acid sequence (SEQ ID NO:2) and corresponding amino acid sequence (SEQ ID NO:3) of a cloned cA2 light chain variable region.

FIG. 16B is a nucleic acid sequence (SEQ ID NO:4) and corresponding amino acid sequence (SEQ ID NO:5) of a cloned cA2 heavy chain variable region.

FIG. 17 is a graphical representation of the early morning stiffness for the five patients in group I, and the four patients in group II is plotted as the mean percent of the baseline value versus time. Both groups showed an approximately 80 percent decrease or greater in early morning stiffness, which persisted for greater than 40 days.

FIG. 18 is a graphical representation of the assessment of pain using a visual analogue scale for the five patients in group I, and the four patients in group II, is plotted as the mean percent of the baseline value versus time. Both groups showed an approximately 60 to 80 percent decrease in pain score which persisted for greater than 40 days.

FIG. 19 is a graphical representation of the Ritchie Articular Index, (a scale scored of joint tenderness), is plotted as the mean percent of the baseline value versus time. Both groups showed an approximately 80 percent decrease in the Ritchie Articular Index, which persisted for greater than 40 days.

FIG. 20 is a graphical representation of the number of swollen joints for the five patients in group I and the four patients in Group II is plotted as the mean percent of baseline value versus time. Both groups showed an approximately 70 to 80 percent decrease in swollen joints, which persisted for 30 to 40 days.

FIG. 21 is a graphical representation of the serum C-reactive protein for four to five patients in group I, and three of the for patients in group II, is plotted as the mean percent of the baseline value versus time. Both groups showed an approximately 80 percent reduction in CRP which persisted for 30 to 40 days. The values for patient number 1 and patient number 7 were omitted from the computations on which the plots are based, since these patients did not have elevated CRP values at baseline.

FIG. 22 is a graphical representation of the erythrocyte sedimentation rate for the five patients in group I and three of the patients in group II is plotted as the mean percent of the baseline value versus time. Both groups showed an approximately 40 percent reduction in ESR which persisted for at least 40 days. The data from patient number 9 is omitted from the computations on which the plots were based, since this patient did not have an elevated ESR at baseline.

FIG. 23 is a graphical representation of the index of Disease Activity, (a composite score of several parameters of disease activity), for the five patients in group I, and the four patients in group II, is plotted as the mean percent of the baseline value versus time. Both groups showed a clinically significant reduction in IDA, which persisted for at least 40 days.

FIG. 24 is a graphical representation of swollen joint counts (maximum 28), as recorded by a

single observer. Circles represent individual patients and horizontal bars show median values at each time point. The screening time point was within 4 weeks of entry to the study (week 0); data from patient 15 were not included after week 2 (dropout). Significance of the changes, relative to week 0, by Mann-Whitney test, adjusted: week 1, $p > 0.05$; week 2, $p < 0.02$; weeks 3-4, $p < 0.002$; weeks 6-8, $p < 0.001$.

FIG. 25 is a graphical representation of levels of serum C-reactive protein (CRP)-Serum CRP (normal range 0-10 mg/liter), measured by nephelometry. Circles represent individual patients and horizontal bars show median values at each time point. The screening time point was within 4 weeks of entry to the study (week 0); data from patient 15 were not included after week 2 (dropout). Significance of the changes, relative to week 0, by Mann-Whitney test, adjusted: week 1, $p < 0.001$; week 2, $p < 0.003$; week 3, $p < 0.002$; week 4, $p < 0.02$; week 6,8, $p < 0.001$.

FIG. 26A is a schematic illustration of the genes encoding TNF receptor/IgG fusion proteins and the gene encoding the truncated light chain. The gene encoding Ig heavy chain (IgH) fusion proteins had the same basic structure as the naturally occurring, rearranged Ig genes except that the Ig variable region coding sequence was replaced with TNF receptor coding sequence. Except for the TNF receptor coding sequences and a partial human K sequence derived by modifying the murine J region coding sequence in the cM-T412 IgH gene by PCT mutagenesis, the entire genomic fragment shown originated from the cM-T412 chimeric mouse/human IgH gene. Looney et al., Hum. Antibody Hybrid. 3: 191-200 (1992). The region deleted in the genes encoding p55-sf3 and p75P-sf3 is marked in the figure. The JC [K]gene, encoding a truncated Ig Kappa light chain, was constructed by deleting the variable region coding sequence from the cM-T412 chimeric mouse/human Ig Kappa gene (Looney, infra) and using PCR mutagenesis to change the murine J sequence to a partial human J sequence. THe p55-light chain fusion in p55-df2 was made by inserting the p55 coding sequence into the EcoRV site in the JC[K]gene. Tracey et al., Nature 330: 662-666 (1987).

FIG. 26B is a schematic illustration of several immunoreceptor molecules of the present invention. The blackened ovals each represent a domain of the IgG1 constant region. THe circles represent the truncated light chain. Small circles adjacent to a p55 or p75 subunit mark the positions of human J sequence. The incomplete circles in p75-sf2 and -sf3 are to illustrate that the C-terminal 53 amino acids of the p75 extracellular domain were deleted. Lines between subunits represent disulfide bonds.

FIG. 27 is a schematic illustration of the construction of a cM-T412 heavy chain so that it has a unique cloning site for insertion of foreign genes such as p55 and p75.

FIG. 28 is a schematic illustration of the construction of the vectors used to express the heavy chain of the immunoreceptors.

FIG. 29 is a schematic illustration of the construction of a cM-T412 light chain so that it has a unique cloning site for insertion of foreign genes such as p55 and p75.

FIG. 30 is a schematic illustration of the construction of the vectors used to express the light chain of the immunoreceptors.

FIGS. 31A-C show graphical representations of fusion proteins protected WEHI 164 cells from TNF beta with actinomycin D and then incubated in 2 ng/ml TNF alpha with varying concentrations of TNF beta overnight at $37o$ C. Cell viability was determined by measuring their uptake of MTT dye. FIG. 31A shows p55 fusion proteins. FIG. 31B shows p75 fusion proteins. FIG. 31C shows comparison of the protective ability of the non-fusion form of p55 (p55-nf) to p55-sf2.

FIG. 32 is a graphical represation of data showing fusion proteins also effectively protect

WEHI 164 cells from TNF beta cytotoxicity.

FIGS. 33A-H are graphical representations of analyses of binding between the various fusion proteins and TNF alpha by saturation binding. (FIGS. 33A and B) and Scatchard analysis (FIGS. 33C-H). A microtiter plate was coated with excess goat anti-Fc polyclonal antibody and incubated with 10 ng/ml of fusion protein in TBST buffer (10 mM Tris-HCI, pH 7.8, 150 mM NaCI, 0.05% Tween-20) for 1 hour. Varying amounts of <125> I labeled TNF alpha (specific activity-34.8 mu Ci/ mu g) was then incubated with the captured fusion protein in PBS (10 mM Na Phosphate, pH 7.0, 150 mM NaCl) with 1% bovine serum albumin for 2 hours. Unbound TNF alpha was washed away with four washes in PBS and the cpm bound was quantitated using a y-counter. All samples were analyzed in triplicate. The slope of the lines in (FIGS. 33C-H) represent the affinity constant, K[a]. The dissociation constant (K[a]) values (see Table I) were derived using the equation K[d] = 1/K[a].

DETDESC:

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

Tumor necrosis factor (TNF) has been discovered to mediate or be involved in many pathologies, such as, but not limited to bacterial, viral or parasitic infections, chronic inflammatory diseases, autoimmune diseases, malignancies, and/or neurodegenerative diseases. Accordingly, anti-TNF compounds and compositions of the present invention which have neutralizing and/or inhibiting activity against TNF are discovered to provide methods for treating and/or diagnosing such pathologies.

The present invention thus provides anti-TNF compounds and compositions comprising anti-TNF antibodies (Abs) and/or anti-TNF peptides which inhibit and/or neutralize TNF biological activity in vitro, in situ and/or in vivo, as specific for association with neutralizing epitopes of human tumor necrosis factor-alpha (hTNF alpha) and/or human tumor necrosis factor beta (hTNF beta). Such anti-TNF Abs or peptides have utilities for use in research, diagnostic and/or therapeutic methods of the present invention for diagnosing and/or treating animals or humans having pathologies or conditions associated with the presence of a substance reactive with an anti-TNF antibody, such as TNF or metabolic products thereof. Such pathologies can include the generalized or local presence of TNF or related compounds, in amounts and/or concentrations exceeding, or less than, those present in a normal healthy subject, or as related to a pathological condition.

Anti-TNF Antibodies and Methods

The term "antibody" is meant to include polyclonal antibodies, monoclonal antibodies (mAbs), chimeric antibodies, anti-idiotypic (anti-Id) antibodies to antibodies that can be labeled in soluble or bound form, as well as fragments, regions or derivatives thereof, provided by any known technique, such as, but not limited to enzymatic cleavage, peptide synthesis or recombinant techniques. Such anti-TNF antibodies of the present invention are capable of binding portions of TNF that inhibit the binding of TNF to TNF receptors.

Polyclonal antibodies are heterogeneous populations of antibody molecules derived from the sera of animals immunized with an antigen. A monoclonal antibody contains a substantially homogeneous population of antibodies specific to antigens, which population contains substantially similar epitope binding sites. MAbs may be obtained by methods known to those skilled in the art. See, for example Kohler and Milstein, Nature 256: 495-497 (1975); U.S. Pat. No. 4,376,110; Ausubel et al, eds., CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, Greene Publishing Assoc. and Wiley Interscience, N.Y., (1987, 1992); and Harlow and Lane ANTIBODIES: A LABORATORY MANUAL Cold Spring Harbor Laboratory (1988); Colligan et al., eds., Current Protocols in Immunology, Greene Publishing Assoc. and Wiley Interscience, N.Y., (1992, 1993), the contents of which references are incoporated entirely herein by

reference. Such antibodies may be of any immunoglobulin class including IgG, IgM, IgE, IgA, GILD and any subclass thereof. A hybridoma producing a mAb of the present invention may be cultivated in vitro, in situ or in vivo. Production of high titers of mAbs in vivo or in situ makes this the presently preferred method of production.

Chimeric antibodies are molecules different portions of which are derived from different animal species, such as those having variable region derived from a murine mAb and a human immunoglobulin constant region, which are primarily used to reduce immunogenicity in application and to increase yields in production, for example, where murine mAbs have higher yields from hybridomas but higher immunogenicity in humans, such that human/murine chimeric mAbs are used. Chimeric antibodies and methods for their production are known in the art (Cabilly et al, Proc. Natl. Acad. Sci. USA 81: 3273-3277 (1984); Morrison et al., Proc. Natl. Acad. Sci. USA 81: 6851-6855 (1984); Boulianne et al., Nature 312: 643-646 (1984); Cabilly et al., European Patent Application 125023 (published Nov. 14, 1984); Neuberger et al., Nature 314: 268-270 (1985); Taniguchi et al., European Patent Application 171496 (published Feb. 19, 1985); Morrison et al., European Patent Application 173494 (published Mar. 5, 1986); Neuberger et al., PCT Application WO 86/01533, (published Mar. 13, 1986); Kudo et al., European Patent Application 184187 (published Jun. 11, 1986); Morrison et al., European Patent Application 173494 (published Mar. 5, 1986); Sahagan et al., J. Immunol. 137: 1066-1074 (1986); Robinson et al., International Patent Publication #PCT/US86/02269 (published 7 May 1987); Liu et al., Proc. Natl. Acad. Sci. USA 84: 3439-3443 (1987); Sun et al., Proc. Natl. Acad. Sci. USA 84: 214-218 (1987); Better et al., Science 240: 1041-1043 (1988); and Harlow and Lane ANTIBODIES: A LABORATORY MANUAL Cold Spring Harbor Laboratory (1988)). These references are entirely incorporated herein by reference.

An anti-idiotypic (anti-Id) antibody is an antibody which recognizes unique determinants generally associated with the antigen-binding site of an antibody. An Id antibody can be prepared by immunizing an animal of the same species and genetic type (e.g., mouse strain) as the source of the mAb with the mAb to which an anti-Id is being prepared. The immunized animal will recognize and respond to the idiotypic determinants of the immunizing antibody by producing an antibody to these idiotypic determinants (the anti-Id antibody). See, for example, U.S. Pat. No. 4,699,880, which is herein entirely incorporated by reference.

The anti-Id antibody may also be used as an "immunogen" to induce an immune response in yet another animal, producing a so-called anti-anti-Id antibody. The anti-anti-Id may be epitopically identical to the original mAb which induced the anti-Id. Thus, by using antibodies to the idiotypic determinants of a mAb, it is possible to identify other clones expressing antibodies of identical specificity.

Anti-TNF antibodies of the present invention can include at least one of a heavy chain constant region (H[c]), a heavy chain variable region (H[v]), a light chain variable region (L [v]) and a light chain constant regions (L[c]), wherein a polyclonal Ab, monoclonal Ab, fragment and/or regions thereof include at least one heavy chain variable region (H[v]) or light chain variable region (L[v]) which binds a portion of a TNF and inhibits and/or neutralizes at least one TNF biological activity.

Preferred antibodies of the present invention are high affinity human-murine chimeric anti-TNF antibodies, and fragments or regions thereof, that have potent inhibiting and/or neutralizing activity in vivo against human TNF alpha . Such antibodies and chimeric antibodies can include those generated by immunization using purified recombinant hTNF alpha (SEQ ID NO:1) or peptide fragments thereof. Such fragments can include epitopes of at least 5 amino acids of residues 87-107, or a combination of both of 59-80 and 87-108 of hTNF alpha (as these corresponding amino acids of SEQ ID NO:1). Additionally, preferred antibodies, fragments and regions of anti-TNF antibodies of the present invention do not recognize amino acids from at least one of amino acids 11-13, 37-42, 49-57 or 155-157 of

hTNF alpha (of SEQ ID NO:1).

Preferred anti-TNF mAbs are also those which will competitively inhibit in vivo the binding to human TNF alpha of anti-TNF alpha murine mAb A2, chimeric mAb cA2, or an antibody having substantially the same specific binding characteristics, as well as fragments and regions thereof. Preferred antibodies of the present invention are those that bind epitopes recognized by A2 and cA2, which are included in amino acids 59-80 and/or 87-108 of hTNF alpha (as these corresponding amino acids of SEQ ID NO:1), such that the epitopes consist of at least 5 amino acids which comprise at least one amino acid from the above portions of human TNF alpha .

Preferred methods for determining mAb specificity and affinity by competitive inhibition can be found in Harlow, et al., Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1988), Colligan et al., eds., Current Protocols in Immunology, Greene Publishing Assoc. and Wiley Interscience, N.Y., (1992, 1993), and Muller, Meth. Enzymol. 92: 589-601 (1983), which references are entirely incorporated herein by reference.

The techniques to raise antibodies of the present invention to small peptide sequences that recognize and bind to those sequences in the free or conjugated form or when presented as a native sequence in the context of a large protein are well known in the art. Such antibodies include murine, murine human and human-human antibodies produced by hybridoma or recombinant techniques known in the art.

As used herein, the term "antigen binding region" refers to that portion of an antibody molecule which contains the amino acid residues that interact with an antigen and confer on the antibody its specificity and affinity for the antigen. The antibody region includes the "framework" amino acid residues necessary to maintain the proper conformation of the antigen-binding residues.

Preferably, the antigen binding region will be of murine origin. In other embodiments, the antigen binding region can be derived from other animal species, in particular rodents such as rabbit, rat or hamster.

The antigen binding region of the chimeric antibody of the present invention is preferably derived from a non-human antibody specific for human TNF. Preferred sources for the DNA encoding such a non-human antibody include cell lines which produce antibody, preferably hybrid cell lines commonly known as hybridomas. A preferred hybridoma is the A2 hybridoma cell line.

An "antigen" is a molecule or a portion of a molecule capable of being bound by an antibody which is additionally capable of inducing an animal to produce antibody capable of binding to an epitope of that antigen. An antigen can have one or more than one epitope. The specific reaction referred to above is meant to indicate that the antigen will react, in a highly selective manner, with its corresponding antibody and not with the multitude of other antibodies which can be evoked by other antigens. Preferred antigens that bind antibodies, fragments and regions of anti-TNF antibodies of the present invention include at least 5 amino acids comprising at least one of amino acids residues 87-108 or both residues 59-80 and 87-108 of hTNF alpha (of SEQ ID NO:1). Preferred antigens that bind antibodies, fragments and regions of anti-TNF antibodies of the present invention do not include amino acids of amino acids 11-13, 37-42, 49-57 or 155-157 of hTNF alpha (SEQ ID NO:1)

Particular peptides which can be used to generate antibodies of the present invention can include combinations of amino acids selected from at least residues 87-108 or both residues 59-80 and 87-108,-which are combined to provide an epitope of TNF that is bound by anti-TNF antibodies, fragments and regions thereof, and which binding provided anti-TNF

biological activity. Such epitopes include at least 1-5 amino acids and less than 22 amino acids from residues 87-108 or each of residues 59-80 and 87-108, which in combination with other amino acids of TNF provide epitopes of at least 5 amino acids in length.

TNF residues 87-108 or both residues 59-80 and 87-108 of TNF (of SEQ ID NO:1), fragments or combinations of peptides containing therein are useful as immunogens to raise antibodies that will recognize peptide sequences presented in the context of the native TNF molecule.

The term "epitope" is meant to refer to that portion of any molecule capable of being recognized by and bound by an antibody at one or more of the Ab's antigen binding region. Epitopes usually consist of chemically active surface groupings of molecules such as amino acids or sugar side chains and have specific three dimensional structural characteristics as well as specific charge characteristics. By "inhibiting and/or neutralizing epitope" is intended an epitope, which, when bound by an antibody, results in loss of biological activity of the molecule or organism containing the epitope, in vivo, in vitro or in situ, more preferably in vivo, including binding of TNF to a TNF receptor.

Epitopes recognized by antibodies, and fragments and regions thereof, of the present invention can include 5 or more amino acids comprising at least one amino acid of each or both of the following amino acid sequences of TNF, which provide a topographical or three dimensional epitope of TNF which is recognized by, and/or binds with anti-TNF activity, an antibody, and fragments, and variable regions thereof, of the present invention:

59-80: Tyr-Ser-Gln-Val-Leu-Phe-Lys-Gly-Gln-Gly-Cys-Pro-Ser-Thr-His-Val-Leu-Leu-Thr-His-Thr-Ile (AA 59-80 of SEQ ID NO:1); and

87-108: Tyr-Gln-Thr-Lys-Val-Asn-Leu-Leu-Ser-Ala-Ile-Lys-Ser-Pro-Cys-Gln-Arg-Glu-Thr-Pro-Glu-Gly (AA 87-108 of SEQ ID NO:1).

Preferred antibodies, fragments and regions of anti-TNF antibodies of the present invention recognize epitopes including 5 amino acids comprising at least one amino acid from amino acids residues 87-108 or both residues 59-80 and 87-108 of hTNF alpha (of SEQ ID NO:1). Preferred antibodies, fragments and regions of anti-TNF antibodies of the present invention do not recognize epitopes from at least one of amino acids 11-13, 37-42, 49-57 or 155-157 of hTNF alpha (of SEQ ID NO:1). In a preferred embodiment, the epitope comprises at least 2 amino acids from residues 87-108 or both residues 59-80 and 87-108 of hTNF alpha (of SEQ ID NO:1). In another preferred embodiment, the epitope comprises at least 3 amino acids from residues 59-80 and 87-108 of hTNF alpha (of SEQ ID NO:1). In another preferred embodiment, the epitope comprises at least 4 amino acids from residues 87-108 or both residues 59-80 and 87-108 of hTNP alpha (of SEQ ID NO:1). In another preferred embodiment, the epitope comprises at least 5 amino acids from residues 87-108 or both residues 59-80 and 87-108 of hTNF alpha (of SEQ ID NO:1). In another preferred embodiment, the epitope comprises at least 6 amino acids from residues 87-108 or both residues 59-80 and 87-108 of hTNF alpha (of SEQ ID NO:1). In another preferred embodiment, the epitope comprises at least 7 amino acids from residues 87-108 or both residues 59-80 and 87-108 of hTNF alpha (of SEQ ID NO:1).

As used herein, the term "chimeric antibody" includes monovalent, divalent or polyvalent immunoglobulins. A monovalent chimeric antibody is a dimer (HL)) formed by a chimeric H chain associated through disulfide bridges with a chimeric L chain. A divalent chimeric antibody is tetramer (H2L2) formed by two HL dimers associated through at least one disulfide bridge. A polyvalent chimeric antibody can also be produced, for example, by employing a C[H]region that aggregates (e.g., from an IgM H chain, or mu chain).

Murine and chimeric antibodies, fragments and regions of the present invention comprise individual heavy (H) and/or light (L) immunoglobulin chains. A chimeric H chain comprises an

antigen binding region derived from the H chain of a non-human antibody specific for TNF, which is linked to at least a portion of a human H chain C region (C[H]), such as CH1 or CH2.

A chimeric L chain according to the present invention, comprises an antigen binding region derived from the L chain of a non-human antibody specific for TNF, linked to at least a portion of a human L chain C region (C[L]).

Antibodies, fragments or derivatives having chimeric H chains and L chains of the same or different variable region binding specificity, can also be prepared by appropriate association of the individual polypeptide chains, according to known method steps, e.g., according to Ausubel infra, Harlow infra, and Colligan infra, the contents of which references are incoporated entirely herein by reference.

With this approach, hosts expressing chimeric H chains (or their derivatives) are separately cultured from hosts expressing chimeric L chains (or their derivatives), and the immunoglobulin chains are separately recovered and then associated. Alternatively, the hosts can be co-cultured and the chains allowed to associate spontaneously in the culture medium, followed by recovery of the assembled immunoglobulin, fragment or derivative.

The hybrid cells are formed by the fusion of a non-human anti-hTNF alpha antibody-producing cell, typically a spleen cell of an animal immunized against either natural or recombinant human TNF, or a peptide fragment of the human TNF alpha protein sequence. Alternatively, the non-human anti-TNF alpha antibody-producing cell can be a B lymphocyte obtained from the blood, spleen, lymph nodes or other tissue of an animal immunized with TNF.

The second fusion partner, which provides the immortalizing function, can be lymphoblastoid cell or a plasmacytoma or myeloma cell, which is not itself an antibody producing cell, but is malignant. Preferred fusion partner cells include the hybridoma SP2/0-Ag14, abbreviated as SP2/0 (ATCC CRL1581) and the myeloma P3X63A98 (ATCC TIB9), or its derivatives. See, e.g, Ausubel infra, Harlow infra, and Colligan infra, the contents of which references are incoporated entirely herein by reference.

Murine hybridomas which produce mAb specific for human TNF alpha or TNF beta are formed by the fusion of a mouse fusion partner cell, such as SP2/0, and spleen cells from mice immunized against purified hTNF alpha , recombinant hTNF alpha , natural or synthetic TNF peptides, including peptides including 5 or more amino acids selected from residues 59-80, and 87-108 of TNF (of SEQ ID NO:1) or other biological preparations containing TNF. To immunize the mice, a variety of different conventional protocols can be followed. For example, mice can receive primary and boosting immunizations of TNF.

The antibody-producing cell contributing the nucleotide sequences encoding the antigen-binding region of the chimeric antibody of the present invention can also be produced by transformation of a non-human, such as a primate, or a human cell. For example, a B lymphocyte which produces anti-TNF antibody can be infected and transformed with a virus such as Epstein-Barr virus to yield an immortal anti-TNF producing cell (Kozbor et al. Immunol. Today 4: 72-79 (1983)). Alternatively, the B lymphocyte can be transformed by providing a transforming gene or transforming gene product, as is well-known in the art. See, e.g, Ausubel infra, Harlow infra, and Colligan infra, the contents of which references are incoporated entirely herein by reference.

Antibody Production Using Hybridomas

The cell fusions are accomplished by standard procedures well known to those skilled in the field of immunology. Fusion partner cell lines and methods for fusing and selecting

hybridomas and screening for mAbs are well known in the art. See, e.g, Ausubel infra, Harlow infra, and Colligan infra, the contents of which references are incoporated entirely herein by reference.

The hTNF alpha -specific murine or chimeric mAb of the present invention can be produced in large quantities by injecting hybridoma or transfectoma cells secreting the antibody into the peritoneal cavity of mice and, after appropriate time, harvesting the ascites fluid which contains a high titer of the mAb, and isolating the mAb therefrom. For such in vivo production of the mAb with a non-murine hybridoma (e.g., rat or human), hybridoma cells are preferably grown in irradiated or athymic nude mice. Alternatively, the antibodies can be produced by culturing hybridoma or transfectoma cells in vitro and isolating secreted mAb from the cell culture medium or recombinantly, in eukaryotic or prokaryotic cells.

In a preferred embodiment, the antibody is a MAb which binds amino acids of an epitope of TNF, which antibody is designated A2, rA2 or cA2, which is produced by a hybridoma or by a recombinant host. In another preferred embodiment, the antibody is a chimeric antibody which recognizes an epitope recognized by A2. In a more preferred embodiment, the antibody is a chimeric antibody designated as chimeric A2 (cA2).

As examples of antibodies according to the present invention, murine mAb A2 of the present invention is produced by a cell line designated c134A. Chimeric antibody cA2 is produced by a cell line designated c168A. Cell line c134A is deposited as a research cell bank in the Centocor Cell Biology Services Depository, and cell line c168A(RCB) is deposited as a research cell bank in the Centocor Corporate Cell Culture Research and Development Depository, both at Centocor, 200 Great Valley Parkway, Malvern, Pa., 19355. The c168A cell line is also deposited at Centocor BV, Leiden, The Netherlands.

The invention also provides for "derivatives" of the murine or chimeric antibodies, fragments, regions or derivatives thereof, which term includes those proteins encoded by truncated or modified genes to yield molecular species functionally resembling the immunoglobulin fragments. The modifications include, but are not limited to, addition of genetic sequences coding for cytotoxic proteins such as plant and bacterial toxins. The fragments and derivatives can be produced from any of the hosts of this invention. Alternatively, anti-TNF antibodies, fragments and regions can be bound to cytotoxic proteins or compounds in vitro, to provide cytotoxic anti-TNF antibodies which would selectively kill cells having TNF receptors.

Fragments include, for example, Fab, Fab', F(ab')2 and Fv. These fragments lack the Fc fragment of intact antibody, clear more rapidly from the circulation, and can have less non-specific tissue binding than an intact antibody (Wahl et al., J. Nucl. Med. 24: 316-325 (1983)). These fragments are produced from intact antibodies using methods well known in the art, for example by proteolytic cleavage with enzymes such as papain (to produce Fab fragments) or pepsin (to produce F(ab')2 fragments).

The identification of these antigen binding region and/or epitopes recognized by mAbs of the present invention provides the information necessary to generate additional monoclonal antibodies with similar binding characteristics and therapeutic or diagnostic utility that parallel the embodiments of this application.

In a preferred embodiment, the amino acids of the epitope are not of at least one of amino acids 11-13, 37-42, 49-57 and 155-157 of hTNF alpha (of SEQ ID NO:1).

Unexpectedly, anti-TNF antibodies or peptides of the present invention can block the action of TNF- alpha without binding to the putative receptor binding locus such as is presented by Eck and Sprang (J. Biol. Chem. 264(29), 17595-17605 (1989), as amino acids 11-13, 37-42, 49-57 and 155-157 of hTNF alpha (of SEQ ID NO:1).

Recombinant Expression of Anti-TNF Antibodies

Recombinant murine or chimeric murine-human or human-human antibodies that inhibit TNF and bind an epitope included in the amino acid sequences residues 87-108 or both residues 59-80 and 87-108 of hTNF alpha (of SEQ ID NO:1), can be provided according to the present invention using known techniques based on the teaching provided herein. See, e.g., Ausubel et al., eds. Current Protocols in Molecular Biology, Wiley Interscience, N.Y. (1987, 1992, 1993); and Sambrook et al. Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press (1989), the entire contents of which are incorporated herein by reference.

The DNA encoding an anti-TNF antibody of the present invention can be genomic DNA or cDNA which encodes at least one of the heavy chain constant region (H[c]), the heavy chain variable region (H[v]), the light chain variable region (L[v]) and the light chain constant regions (L[c]). A convenient alternative to the use of chromosomal gene fragments as the source of DNA encoding the murine V region antigen-binding segment is the use of cDNA for the construction of chimeric immunoglobulin genes, e.g., as reported by Liu et al. (Proc. Natl. Acad. Sci., USA 84: 3439 (1987) and J. Immunology 139: 3521 (1987), which references are hereby entirely incorporated herein by reference. The use of cDNA requires that gene expression elements appropriate for the host cell be combined with the gene in order to achieve synthesis of the desired protein. The use of cDNA sequences is advantageous over genomic sequences (which contain introns), in that cDNA sequences can be expressed in bacteria or other hosts which lack appropriate RNA splicing systems.

For example, a cDNA encoding a murine V region antigen-binding segment having anti-TNF activity can be provided using known methods based on the use of the DNA sequence presented in FIG. 16A (SEQ ID NO:2). Alternatively, a cDNA encoding a murine C region antigen-binding segment having anti-TNF activity can be provided using known methods based on the use of the DNA sequence presented in FIG. 16B (SEQ ID NO:3). Probes that bind a portion of the DNA sequence presented in FIG. 16B or 17B can be used to isolate DNA from hybridomas expressing TNF antibodies, fragments or regions, as presented herein, according to the present invention, by known methods.

Oligonucleotides representing a portion of the variable region presented in FIG. 16A or 16B sequence are useful for screening for the presence of homologous genes and for the cloning of such genes encoding variable or constant regions of an anti-TNF antibody. Such probes preferably bind to portions of sequences according to FIGS. 16A or 16B which encode light chain or heavy chain variable regions which bind an activity inhibiting epitope of TNF, especially an epitope of at least 5 amino acids of residues 87-108 or a combination of residues 59-80 and 87-108 (of SEQ ID NO:1).

Such techniques for synthesizing such oligonucleotides are well known and disclosed by, for example, Wu, et al., Prog. Nucl. Acid. Res. Molec. Biol. 21: 101-141 (1978)), and Ausubel et al, eds. Current Protocols in Molecular Biology, Wiley Interscience (1987, 1993), the entire contents of which are herein incorporated by reference.

Because the genetic code is degenerate, more than one codon can be used to encode a particular amino acid (Watson, et al., infra). Using the genetic code, one or more different oligonucleotides can be identified, each of which would be capable of encoding the amino acid. The probability that a particular oligonucleotide will, in fact, constitute the actual XXX-encoding sequence can be estimated by considering abnormal base pairing relationships and the frequency with which a particular codon is actually used (to encode a particular amino acid) in eukaryotic or prokaryotic cells expressing an anti-TNF antibody or fragment. Such "codon usage rules" are disclosed by Lathe, et al., J. Molec. Biol. 183: 1-12 (1985). Using the "codon usage rules" of Lathe, a single oligonucleotide, or a set of oligonucleotides, that contains a theoretical "most probable" nucleotide sequence capable of encoding anti-TNF

variable or constant region sequences is identified.

Although occasionally an amino acid sequence can be encoded by only a single oligonucleotide, frequently the amino acid sequence can be encoded by any of a set of similar oligonucleotides. Importantly, whereas all of the members of this set contain oligonucleotides which are capable of encoding the peptide fragment and, thus, potentially contain the same oligonucleotide sequence as the gene which encodes the peptide fragment, only one member of the set contains the nucleotide sequence that is identical to the nucleotide sequence of the gene. Because this member is present within the set, and is capable of hybridizing to DNA even in the presence of the other members of the set, it is possible to employ the unfractionated set of oligonucleotides in the same manner in which one would employ a single oligonucleotide to clone the gene that encodes the protein.

The oligonucleotide, or set of oligonucleotides, containing the theoretical "most probable" sequence capable of encoding an anti-TNF antibody or fragment including a variable or constant region is used to identify the sequence of a complementary oligonucleotide or set of oligonucleotides which is capable of hybridizing to the "most probable" sequence, or set of sequences. An oligonucleotide containing such a complementary sequence can be employed as a probe to identify and isolate the variable or constant region anti-TNF gene (Sambrook et al., infra).

A suitable oligonucleotide, or set of oligonucleotides, which is capable of encoding a fragment of the variable or constant anti-TNF region (or which is complementary to such an oligonucleotide, or set of oligonucleotides) is identified (using the above-described procedure), synthesized, and hybridized by means well known in the art, against a DNA or, more preferably, a cDNA preparation derived from cells which are capable of expressing anti-TNF antibodies or variable or constant regions thereof. Single stranded oligonucleotide molecules complementary to the "most probable" variable or constant anti-TNF region peptide coding sequences can be synthesized using procedures which are well known to those of ordinary skill in the art (Belagaje, et al., J. Biol. Chem. 254: 5765-5780 (1979); Maniatis, et al., In: Molecular Mechanisms in the Control of Gene Expression, Nierlich, et al., Eds., Acad. Press, NY (1976); Wu, et al., Prog. Nucl. Acid Res. Molec. Biol. 21: 101-141 (1978); Khorana, Science 203: 614-625 (1979)). Additionally, DNA synthesis can be achieved through the use of automated synthesizers. Techniques of nucleic acid hybridization are disclosed by Sambrook et al. (infra), and by Haymes, et al. (In: Nucleic Acid Hybridization, A Practical Approach, IRL Press, Washington, D.C. (1985)), which references are herein incorporated by reference. Techniques such as, or similar to, those described above have successfully enabled the cloning of genes for human aldehyde dehydrogenases (Hsu, et al., Proc. Natl. Acad. Sci. USA 82: 3771-3775 (1985)), fibronectin (Suzuki, et al., Eur. Mol. Biol. Organ. J. 4: 2519-2524 (1985)), the human estrogen receptor gene (Walter, et al., Proc. Natl. Acad. Sci. USA 82: 7889-7893 (1985)), tissue-type plasminogen activator (Pennica, et al., Nature 301: 214-221 (1983)) and human term placental alkaline phosphatase complementary DNA (Kam, et al., Proc. Natl. Acad. Sci. USA 82: 8715-8719 (1985)) .

In an alternative way of cloning a polynucleotide encoding an anti-TNF variable or constant region, a library of expression vectors is prepared by cloning DNA or, more preferably, cDNA (from a cell capable of expressing an anti-TNF antibody or variable or constant region) into an expression vector. The library is then screened for members capable of expressing a protein which competitively inhibits the binding of an anti-TNF antibody, such as A2 or cA2, and which has a nucleotide sequence that is capable of encoding polypeptides that have the same amino acid sequence as anti-TNF antibodies or fragments thereof. In this embodiment, DNA, or more preferably cDNA, is extracted and purified from a cell which is capable of expressing an anti-TNF antibody or fragment. The purified cDNA is fragmentized (by shearing, endonuclease digestion, etc.) to produce a pool of DNA or cDNA fragments. DNA or cDNA fragments from this pool are then cloned into an expression vector in order to produce

a genomic library of expression vectors whose members each contain a unique cloned DNA or cDNA fragment such as in a lambda phage library, expression in prokaryotic cell (e.g., bacteria) or eukaryotic cells, (e.g., mammalian, yeast, insect or fungus). See, e.g., Ausubel, infra, Harlow, infra, Colligan, infra; Nyyssonen et al. Bio/Technology 11: 591-595 (Can 1993); Marks et al., Bio/Technology 11: 1145-1149 (October 1993). Once nucleic acid encoding such variable or constant anti-TNF regions is isolated, the nucleic acid can be appropriately expressed in a host cell, along with other constant or variable heavy or light chain encoding nucleic acid, in order to provide recombinant MAbs that bind TNF with inhibitory activity. Such antibodies preferably include a murine or human anti-TNF variable region which contains a framework residue having complimentarily determining residues which are responsible for antigen binding. In a preferred embodiment, an anti-TNF variable light or heavy chain encoded by a nucleic acid as described above binds an epitope of at least 5 amino acids including residues 87-108 or a combination of residues 59-80 and 87-108 of hTNF (of SEQ ID NO:1).

Human genes which encode the constant (C) regions of the murine and chimeric antibodies, fragments and regions of the present invention can be derived from a human fetal liver library, by known methods. Human C regions genes can be derived from any human cell including those which express and produce human immunoglobulins. The human C[H]region can be derived from any of the known classes or isotypes of human H chains, including gamma, mu , alpha , delta or epsilon , and subtypes thereof, such as G1, G2, G3 and G4. Since the H chain isotype is responsible for the various effector functions of an antibody, the choice of C[H]region will be guided by the desired effector functions, such as complement fixation, or activity in antibody-dependent cellular cytotoxicity (ADCC). Preferably, the C[H] region is derived from gamma 1 (IgG1), gamma 3 (IgG3), gamma 4 (IgG4), or mu (IgM).

The human C[L]region can be derived from either human L chain isotype, kappa or lambda.

Genes encoding human immunoglobulin C regions are obtained from human cells by standard cloning techniques (Sambrook, et al. (Molecular Cloning: A Laboratory Manual, 2nd Edition, Cold Spring Harbor Press, Cold Spring Harbor, N.Y. (1989) and Ausubel et al, eds. Current Protocols in Molecular Biology (1987-1993)). Human C region genes are readily available from known clones containing genes representing the two classes of L chains, the five classes of H chains and subclasses thereof. Chimeric antibody fragments, such as F(ab')2 and Fab, can be prepared by designing a chimeric H chain gene which is appropriately truncated. For example, a chimeric gene encoding an H chain portion of an F(ab')2 fragment would include DNA sequences encoding the CH1 domain and hinge region of the H chain, followed by a translational stop codon to yield the truncated molecule.

Generally, the murine, human or murine and chimeric antibodies, fragments and regions of the present invention are produced by cloning DNA segments encoding the H and L chain antigen-binding regions of a TNF-specific antibody, and joining these DNA segments to DNA segments encoding C[H]and C[L]regions, respectively, to produce murine, human or chimeric immunoglobulin-encoding genes.

Thus, in a preferred embodiment, a fused chimeric gene is created which comprises a first DNA segment that encodes at least the antigen-binding region of non-human origin, such as a functionally rearranged V region with joining (J) segment, linked to a second DNA segment encoding at least a part of a human C region.

Therefore, cDNA encoding the antibody V and C regions, the method of producing the chimeric antibody according to the present invention involves several steps, outlined below:

1. isolation of messenger RNA (mRNA) from the cell line producing an anti-TNF antibody and from optional additional antibodies supplying heavy and light constant regions; cloning and cDNA production therefrom;

2. preparation of a full length cDNA library from purified mRNA from which the appropriate V and/or C region gene segments of the L and H chain genes can be: (i) identified with appropriate probes, (ii) sequenced, and (iii) made compatible with a C or V gene segment from another antibody for a chimeric antibody;

3. Construction of complete H or L chain coding sequences by linkage of the cloned specific V region gene segments to cloned C region gene, as described above;

4. Expression and production of L and H chains in selected hosts, including prokaryotic and eukaryotic cells to provide murine-murine, human-murine, human-human or human murine antibodies.

One common feature of all immunoglobulin H and L chain genes and their encoded mRNAs is the J region. H and L chain J regions have different sequences, but a high degree of sequence homology exists (greater than 80%) among each group, especially near the C region. This homology is exploited in this method and consensus sequences of H and L chain J regions can be used to design oligonucleotides for use as primers for introducing useful restriction sites into the J region for subsequent linkage of V region segments to human C region segments.

C region cDNA vectors prepared from human cells can be modified by site-directed mutagenesis to place a restriction site at the analogous position in the human sequence. For example, one can clone the complete human kappa chain C (C[k]) region and the complete human gamma-1 C region (C[gamma-1]). In this case, the alternative method based upon genomic C region clones as the source for C region vectors would not allow these genes to be expressed in bacterial systems where enzymes needed to remove intervening sequences are absent. Cloned V region segments are excised and ligated to L or H chain C region vectors. Alternatively, the human C[gamma-1]region can be modified by introducing a termination codon thereby generating a gene sequence which encodes the H chain portion of an Fab molecule. The coding sequences with linked V and C regions are then transferred into appropriate expression vehicles for expression in appropriate hosts, prokaryotic or eukaryotic.

Two coding DNA sequences are said to be "operably linked" if the linkage results in a continuously translatable sequence without alteration or interruption of the triplet reading frame. A DNA coding sequence is operably linked to a gene expression element if the linkage results in the proper function of that gene expression element to result in expression of the coding sequence.

Expression vehicles include plasmids or other vectors. Preferred among these are vehicles carrying a functionally complete human C[H]or C[L]chain sequence having appropriate restriction sites engineered so that any V[H]or V[L]chain sequence with appropriate cohesive ends can be easily inserted therein. Human C[H]or C[L]chain sequence-containing vehicles thus serve as intermediates for the expression of any desired complete H or L chain in any appropriate host.

A chimeric antibody, such as a mouse-human or human-human, will typically be synthesized from genes driven by the chromosomal gene promoters native to the mouse H and L chain V regions used in the constructs; splicing usually occurs between the splice donor site in the mouse J region and the splice acceptor site preceding the human C region and also at the splice regions that occur within the human C[H]region; polyadenylation and transcription termination occur at native chromosomal sites downstream of the human coding regions.

Non-Limiting Exemplary Chimetic A2 (cA2) Anti-TNF Antibody of the Present Invention

Murine MAbs are undesirable for human therapeutic use, due to a short free circulating serum half-life and the stimulation of a human anti-murine antibody (HAMA) response. A murine-human chimeric anti-human TNF alpha MAb was developed in the present invention with high affinity, epitope specificity and the ability to neutralize the cytotoxic effects of human TNF. Chimeric A2 anti-TNF consists of the antigen binding variable region of the high-affinity neutralizing mouse anti-human TNF IgG1 antibody, designated A2, and the constant regions of a human IgG1, kappa immunoglobulin. The human IgG1 Fc region is expected to: improve allogeneic antibody effector function; increase the circulating serum half-life; and decrease the immunogenicity of the antibody. A similar murine-human chimeric antibody (chimeric 17-1A) has been shown in clinical studies to have a 6-fold longer in vivo circulation time and to be significantly less immunogenic than its corresponding murine MAb counterpart (LoBuglio et al., Proc Natl Acad Sci USA 86: 4220-4224, 1988).

The avidity and epitope specificity of the chimeric A2 is derived from the variable region of the murine A2. In a solid phase ELISA, cross-competition for TNF was observed between chimeric and murine A2, indicating an identical epitope specificity of cA2 and murine A2. The specificity of cA2 for TNF- alpha was confirmed by its inability to neutralize the cytotoxic effects of lymphotoxin (TNF- beta). Chimeric A2 neutralizes the cytotoxic effect of both natural and recombinant human TNF in a dose dependent manner. From binding assays of cA2 and recombinant human TNF, the affinity constant of cA2 was calculated to be 1.8 x 10^9 M^{-1} .

ANTI-TNF Immunoreceptor Peptides

Immunoreceptor peptides of this invention can bind to TNF alpha and/or TNF beta . The immunoreceptor comprises covalently attached to at least a portion of the TNF receptor at least one immunoglobulin heavy or light chain. In certain preferred embodiments, the heavy chain constant region comprises at least a portion of CH1. Specifically, where a light chain is included with an immunoreceptor peptide, the heavy chain must include the area of CH1 responsible for binding a light chain constant region.

An immunoreceptor peptide of the present invention can preferably comprise at least one heavy chain constant region and, in certain embodiments, at least one light chain constant region, with a receptor molecule covalently attached to at least one of the immunoglobulin chains. Light chain or heavy chain variable regions are included in certain embodiments. Since the receptor molecule can be linked within the interior of an immunoglobulin chain, a single chain can have a variable region and a fusion to a receptor molecule.

The portion of the TNF receptor linked to the immunoglobulin molecule is capable of binding TNF alpha and/or TNF beta . Since the extracellular region of the TNF receptor binds TNF, the portion attached to the immunoglobulin molecule of the immunoreceptor consists of at least a portion of the extracellular region of the TNF receptor. In certain preferred embodiments, the entire extracellular region of p55 is included. In other preferred embodiments, the entire extracellular region of p75 is included. In further preferred embodiments, the extracellular region of p75 is truncated to delete at least a portion of a region of O-linked glycosylation and/or a proline-rich region while leaving intact the intramolecular disulfide bridges. Such immunoreceptors comprise at least a portion of a hinge region wherein at least one heavy chain is covalently linked to a truncated p75 extracellular region capable of binding to TNF alpha or TNF beta or both. Such a truncated molecule includes, for example, sequences 1-178, 1-182 or at least 5 amino acid portions thereof, such as 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, . . . 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 210, 220, 230, 240, 250, 260, 270, 280, 290 or any value thereon.

Certain embodiments can also include, for example, the C-terminal half of the hinge region to provide a disulfide bridge between heavy chains where both CH2 and CH3 chains are present

and CH1 is absent. Alternatively, for example, the N-terminal half of the hinge region can be included to provide a disulfide bridge with a light chain where only the CH1 region is present.

In certain preferred embodiments of this invention, the non-immunoglobulin molecule is covalently linked to the N-terminus of at least one CH1 region. In other preferred embodiments, the non-immunoglobulin molecule is covalently linked to an interior section of at least one heavy and/or light chain region. Thus, a portion of the TNF receptor can be, for example, at the end of the immunoglobulin chain or in the middle of the chain.

Where the TNF receptor is attached to the middle of the immunoglobulin, the immunoglobulin chain can be truncated, for example, to compensate for the presence of foreign amino acids, thus resulting in a fusion molecule of approximately the same length as a natural immunoglobulin chain. Alternatively, for example, the immunoglobulin chain can be present substantially in its entirety, thus resulting in a chain that is longer than the corresponding natural immunoglobulin chain. Additionally, the immunoglobulin molecule can be truncated to result in a length intermediate between the size of the entire chain linked to the receptor molecule and the size of the immunoglobulin chain alone.

In certain preferred embodiments, the heavy chain is an IgG class heavy chain. In other preferred embodiments, the heavy chain is an IgM class heavy chain.

In certain preferred embodiments, the heavy chain further comprises at least about 8 amino acids of a J region.

In certain preferred embodiments, at least a portion of the hinge region is attached to the CH1 region. For example, where CH1 and CH2 are present in the molecule, the entire hinge region is also present to provide the disulfide bridges between the two heavy chain molecules and between the heavy and light chains. Where only CH1 is present, for example, the molecule need only contain the portion of the hinge region corresponding to the disulfide bridge between the light and heavy chains, such as the first 7 amino acids of the hinge.

It will be understood by one skilled in the art, once armed with the present disclosure, that the immunoreceptor peptides of the invention can be, for example, monomeric or dimeric. For example, the molecules can have only one light chain and one heavy chain or two light chains and two heavy chains.

At least one of the non-immunoglobulin molecules linked to an immunoglobin molecule comprises at least a portion of p55 or at least a portion of p75. The portion of the receptor that is included encompasses the TNF binding site.

In certain preferred embodiments, the non-immunoglobulin molecule comprises at least 5 amino acid segments of sequences 2-159 of p55. In other preferred embodiments, the non-immunoglobulin molecule comprises at least 5 amino acid portions of sequences 1-235 of p75. In further preferred embodiments, the non-immunoglobulin molecule comprises at least 5 amino acid portions of sequences 1-182 of p75. The above 5 amino acid portions can be selected from 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, . . . 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 210, 220, 230, 240, 250, 260, 270, 280, 290.

In certain preferred embodiments, each of the two heavy chains and each of the two light chains is linked to a portion of the TNF receptor, thus forming a tetravalent molecule. Such a tetravalent molecule can have, for example, four p55 receptor molecules; two on the two heavy chains and two on the two light chains. Alternatively, a tetravalent molecule can have, for example, a p55 receptor molecule attached to each of the two heavy chains and a p75 receptor molecule attached to each of the two light chains. A tetravalent molecule can also

have, for example, p55 receptor attached to the light chains and p75 receptor attached to the heavy chains. Additionally, a tetravalent molecule can have one heavy chain attached to p55, one heavy chain attached to p75, one light chain attached to p75, and one light chain attached to p55. See, for example, the molecules depicted in FIG. 26A. Further, the molecules can have six receptors attached, for example; two within the heavy chains and four at the ends of the heavy and light chains. Other potential multimers and combinations would also be within the scope of one skilled in the art, once armed with the present disclosure.

In further preferred embodiments, at least one of the heavy chains has a variable region capable of binding to a second target molecule. Such molecules include, for example, CD3, so that one half of a fusion molecule is a monomeric anti-CD3 antibody.

Additionally, in other embodiments of the present invention, the immunoreceptor peptides further include an irrelevant variable region on the light chain and/or heavy chain. Preferably, however, such a region is absent due to the lowered affinity for TNF which can be present due to stearic hindrance.

In certain preferred embodiments, the heavy chain is linked to a non-immunoglobulin molecule capable of binding to a second target molecule, such as a cytotoxic protein, thus creating a part immunoreceptor, part immunotoxin that is capable of killing those cells expressing TNF. Such cytotoxic proteins, include, but are not limited to, Ricin-A, Pseudomonas toxin, Diphtheria toxin and TNF. Toxins conjugated to ligands are known in the art (see, for example, Olsnes, S. et al., Immunol. Today 1989, 10, 291-295). Plant and bacterial toxins typically kill cells by disruption the protein synthetic machinery.

The Immunoreceptors of this invention can be conjugated to additional types of therapeutic moieties including, but not limited to, radionuclides, cytotoxic agents and drugs. Examples of radionuclides include <212> Bi, <131> I, <186> Re, and <90> Y, which list is not intended to be exhaustive. The radionuclides exert their cytotoxic effect by locally irradiating the cells, leading to various intracellular lesions, as is known in the art of radiotherapy.

Cytotoxic drugs which can be conjugated to the immunoreceptors and subsequently used for in vivo therapy include, but are not limited to, daunorubicin, doxorubicin, methotrexate, and Mitomycin C. Cytotoxic drugs interfere with critical cellular processes including DNA, RNA, and protein synthesis. For a fuller exposition of these classes of drugs which are known in the art, and their mechanisms of action, see Goodman, A. G., et al., Goodman and Gilman's The Pharmacological Basis of Therapeutics, 8th Ed., Macmillan Publishing Col, 1990. Katzung, ed., Basic and Clinical Pharmacology, Fifth Edition, p 768-769, 808-809, 896, Appleton and Lange, Norwalk, Conn.

In preferred embodiments, immunoreceptor molecules of the invention are capable of binding with high affinity to a neutralizing epitope of human TNF alpha or TNF beta in vivo. Preferably, the binding affinity is at least about 1.6×10^{10} M-1. See, for example, Table 1 below. Additionally, in preferred embodiments, immunoreceptor molecules of the invention are capable of neutralizing TNF at an efficiency of about a concentration of less than 130 pM to neutralize 39.2 pM human TNF alpha . See, for example, Table 1.

TABLE 1

Summary of affinities of different fusion proteins for TNF alpha .

protein	IC50*	Molar ration fp: TNF alpha at IC50	K[D] (pM)
p55-sf2	70	1.8	57
p55-df2	55	1.4	60
p55-sf3	100	2.6	48

p75-sf2	130 ·	3.3	33
p75P-sf2	70	1.8 ·	29
p75P-sf3	130	3.3	15

n*IC50 = concentration of fusion protein required to inhibit 2 ng/ml (39.2 pM) TNF alpha by 50%.62 -

Once armed with the present disclosure, one skilled in the art would be able to create fragments of the immunoreceptor peptides of the invention. Such fragments are intended to be within the scope off this invention. For example, once the molecules are isolated, they can be cleaved with protease to generate fragments that remain capable of binding TNF.

Once armed with the present disclosure, one skilled in the art would also be able to create derivatives of the immunoreceptor peptides of the invention. Such derivatives are intended to be within the scope of this invention. For example, amino acids in the immunoreceptor that constitute a protease recognition site can be modified to avoid protease cleavage and thus confer greater stability, such as KEX2 sites.

One skilled in the art, once armed with the present disclosure, would be able to synthesize the molecules of the invention. The immunoreceptor peptides can be constructed, for example, by vector-mediated synthesis, as described in Example XXIV. In general, two expression vectors are preferably used; one for the heavy chain, one for the light chain. A vector for expression an immunoglobulin preferably consists of a promoter linked to the signal sequence, followed by the constant region. The vector additionally preferably contains a gene providing for the selection of transfected cells expressing the construct. In certain preferred embodiments, sequences derived from the J region are also included.

The immunoglobulin gene can be from any vertebrate source, such as murine, but preferably, it encodes an immunoglobulin having a substantial number of sequences that are of the same origin as the eventual recipient of the immunoreceptor peptide. For example, if a human is treated with a molecule of the invention, preferably the immunoglobulin is of human origin.

TNF receptor constructs for linking to the heavy chain can be synthesized, for example, using DNA encoding amino acids present in the cellular domain of the receptor. Putative receptor binding loci of hTNF have been presented by Eck and Sprange, J. Biol. Chem. 1989, 264(29), 17595-17605, who identified the receptor binding loci of TNF- alpha as consisting of amino acids 11-13, 37-42, 49-57 and 155-157. PCT application WO91/02078 (priority date of Aug. 7, 1989) discloses TNF ligands which can bind to monoclonal antibodies having the following epitopes of at least one of 1-20, 56-77, and 108-127; at least two of 1-20, 56-77, 108-127 and 138-149; all of 1-18, 58-65, 115-125 and 138-149; all of 1-18, and 108-128; all of 56-79, 110-127 and 135- or 136-155; all of 1-30, 117-128 and 141-153; all of 1-26, 117-128 and 141-153; all of 22-40, 49-96 or -97, 110-127 and 136-153; all of 12-22, 36-45, 96-105 and 132-157; all of both of 1-20 and 76-90; all of 22-40, 69-97, 105-128 and 135-155; all of 22-31 and 146-157; all of 22-40 and 49-98; at least one of 22-40, 49-98 and 69-97, both of 22-40 and 70-87. Thus, one skilled in the art, once armed with the present disclosure, would be able to create TNF receptor fusion proteins using portions of the receptor that are known to bind TNF.

Advantages of using an immunoglobulin fusion protein (immunoreceptor peptide) of the present invention include one or more of (1) possible increased avidity for multivalent ligands due to the resulting bivalency of dimeric fusion proteins, (2) longer serum half-life, (3) the

ability to activate effector cells via the Fc domain, (4) ease of purification (for example, by protein A chromatography), (5) affinity for TNF alpha and TNF beta and (6) the ability to block TNF alpha or TNF beta cytotoxicity.

TNF receptor/IgG fusion proteins have shown greater affinity for TNF alpha in vitro than their monovalent, non-fusion counterparts. These types of fusion proteins, which also bind murine TNF with high affinity, have also been shown to protect mice from lipopolysaccharide-induced endotoxemia. Lesslauer et all, Eur. J. Immunol. 1991, 21, 2883-2886; and Ashkenazi et al., Proc. Natl. Acad. Sci. USA 1991, 88, 10535-10539. Unlike the molecules of the present invention, the TNF receptor/IgG fusion proteins reported to date have had the receptor sequence fused directly to the hinge domain of IgGs such that the first constant domain (CH1) of the heavy chain was omitted. Lesslauer et al., Eur. J. Immunol. 1991, 21, 2883-2886; Ashkenzi et al., Proc. Natl. Acad. Sci. USA 1991, 88, 10535-10539; and Peppel et al., J. Exp. Med. 1991, 174, 1483-1489.

While this generally permits secretion of the fusion protein in the absence of an Ig light chain, a major embodiment of the present invention provides for the inclusion of the CH1 domain, which can confer advantages such as (1) increased distance and/or flexibility between two receptor molecules resulting in greater affinity for TNF, (2) the ability to create a heavy chain fusion protein and a light chain fusion protein that would assemble with each other and dimerize to form a tetravalent (double fusion) receptor molecule, and (3) a tetravalent fusion protein can have increased affinity and/or neutralizing capability for TNF compared to a bivalent (single fusion) molecule.

Unlike other TNF receptor/IgG fusion proteins that have been reported, the fusion proteins of a major embodiment of the present invention include the first constant domain (CH1) of the heavy chain. The CH1 domain is largely responsible for interactions with light chains. The light chain, in turn, provides a vehicle for attaching a second set of TNF receptor molecules to the immunoreceptor peptide.

It was discovered using the molecules of the present invention that the p55/light chain fusion proteins and p55/heavy chain fusion proteins would assemble with each other and dimerize to form an antibody-like molecule that is tetravalent with respect to p55. The resulting tetravalent p55 molecules can confer more protection against, and have greater affinity for, TNF alpha or TNF beta than the bivalent p55 molecules. Despite the presumed close proximity of the two light chain p55 domains to the heavy chain p55 domains, they do not appear to stereocilia hinder or reduce the affinity for TNF.

Inclusion of the CH1 domain also meant that secretion of the fusion protein was likely to be inefficient in the absence of light chain. This has been shown to be due to a ubiquitous immunoglobulin binding protein (BiP) that binds to the C[H]1 domain of heavy chains that are not assembled with a light chain and sequesters them in the endoplasmic reticulum. Karlsson et al., J. Immunol. Methods 1991, 145, 229-240.

In initial experiments, an irrelevant light chain was co-transfected with the p55-heavy chain construct and subsequent analyses showed that the two chains did assemble and that the resulting fusion protein protected WEHI cells from TNF alpha . However, it was considered likely that the variable region of the irrelevant light chain would stereocilia hinder interactions between the p55 subunits and TNF alpha . For this reason, a mouse-human chimeric antibody light chain gene was engineered by (1) deleting the variable region coding sequence, and (2) replacing the murine J coding sequence with human J coding sequence. Use of this truncated light chain, which was shown to assemble and disulfide bond with heavy chains, increased the efficiency of TNF inhibition by approximately 30-fold compared to use of a complete irrelevant light chain.

Comparison of the abilities of p75-sf2 and p75P-sf2 to inhibit TNF cytotoxicity indicated that

the C-terminal 53 amino acids of the extracellular domain of p75, which defines a region that is rich in proline residues and contains the only sites of O-linked glycosylation, are not necessary for high-affinity binding to TNF alpha or TNF beta . In fact, the p75P-sf2 construct repeatedly showed higher affinity binding to TNF beta than p75-sf2. Surprisingly, there was no difference observed between the two constructs in their affinity for TNF alpha .

It is possible that a cell-surface version of p75-P would also bind TNF beta with higher affinity than the complete p75 extracellular domain. A similar region is found adjacent to the transmembrane domain in the low affinity nerve growth factor receptor whose extracellular domain shows the same degree of similarity to p75 as p55 does. Mallett et al., Immunol. Today 1991, 12, 220-223.

Two groups have reported that in cell cytotoxicity assays, their p55 fusion protein could be present at a 3-fold (Lesslauer et al., Eur. J. Immunol. 1991, 21, 2883-2886) or 6-8 fold (Ashkenazi et al., Proc. Nat. Acad. Sci. USA 1991, 88, 10535-10539) lower concentration than their monovalent p55 and still get the same degree of protection, while another group (Peppel et al., J. Exp. Med. 1991, 174, 1483-1489) showed that their p55 fusion protein could be present at a 1000-fold lower concentration than monomeric p55. Thus, the prior art has shown unpredictability in the great variability in the efficiency of different fusion proteins.

The molecules of the present invention have demonstrated the same degree of protection against TNF in a 5000-fold lower molar concentration than monomeric p55. (See Table 1.) It is believed that the presence of the CH1 chain in the molecules of a major embodiment of the present invention can confer greater flexibility to the molecule and avoid stearic hindrance with the binding of the TNF receptor.

Recombinant Expression of Anti-TNF Antibodies and Anti-TNF Peptides.

A nucleic acid sequence encoding at least one anti-TNF peptide or Ab fragment of the present invention may be recombined with vector DNA in accordance with conventional techniques, including blunt-ended or staggered-ended termini for ligation, restriction enzyme digestion to provide appropriate termini, filling in of cohesive ends as appropriate, alkaline phosphatase treatment to avoid undesirable joining, and ligation with appropriate ligases. Techniques for such manipulations are disclosed, e.g., by Ausubel, infra, Sambrook, infra, entirely incorporated herein by reference, and are well known in the art.

A nucleic acid molecule, such as DNA, is said to be "capable of expressing" a polypeptide if it contains nucleotide sequences which contain transcriptional and translational regulatory information and such sequences are "operably linked" to nucleotide sequences which encode the polypeptide. An operable linkage is a linkage in which the regulatory DNA sequences and the DNA sequence sought to be expressed are connected in such a way as to permit gene expression as anti-TNF peptides or Ab fragments in recoverable amounts. The precise nature of the regulatory regions needed for gene expression may vary from organism to organism, as is well known in the analogous art. See, e.g., Sambrook, supra and Ausubel supra.

The present invention accordingly encompasses the expression of an anti-TNF peptide or Ab fragment, in either prokaryotic or eukaryotic cells, although eukaryotic expression is preferred.

Preferred hosts are bacterial or eukaryotic hosts including bacteria, yeast, insects, fungi, bird and mammalian cells either in vivo, or in situ, or host cells of mammalian, insect, bird or yeast origin. It is preferred that the mammalian cell or tissue is of human, primate, hamster, rabbit, rodent, cow, pig, sheep, horse, goat, dog or cat origin, but any other mammalian cell may be used.

Further, by use of, for example, the yeast ubiquitin hydrolase system, in vivo synthesis of ubiquitin-transmembrane polypeptide fusion proteins may be accomplished. The fusion proteins so produced may be processed in vivo or purified and processed in vitro, allowing synthesis of an anti-TNF peptide or Ab fragment of the present invention with a specified amino terminus sequence. Moreover, problems associated with retention of initiation codon-derived methionine residues in direct yeast (or bacterial) expression may be avoided. Sabin et al., Bio/Technol. 7(7): 705-709 (1989); Miller et al., Bio/Technol. 7(7): 698-704 (1989).

Any of a series of yeast gene expression systems incorporating promoter and termination elements from the actively expressed genes coding for glycolytic enzymes produced in large quantities when yeast are grown in mediums rich in glucose can be utilized to obtain anti-TNF peptides or Ab fragments of the present invention. Known glycolytic genes can also provide very efficient transcriptional control signals. For example, the promoter and terminator signals of the phosphoglycerate kinase gene can be utilized.

Production of anti-TNF peptides or Ab fragments or functional derivatives thereof in insects can be achieved, for example, by infecting the insect host with a baculovirus engineered to express transmembrane polypeptide by methods known to those of skill. See Ausubel et al, eds. Current Protocols in Molecular Biology, Wiley Interscience, §§16.8-16.11 (1987, 1993).

In a preferred embodiment, the introduced nucleotide sequence will be incorporated into a plasmid or viral vector capable of autonomous replication in the recipient host. Any of a wide variety of vectors may be employed for this purpose. See, e.g., Ausubel et al, infra, §§1.5, 1.10, 7.1, 7.3, 8.1, 9.6, 9.7, 13.4, 16.2, 16.6, and 16.8-16.11. Factors of importance in selecting a particular plasmid or viral vector include: the ease with which recipient cells that contain the vector may be recognized and selected from those recipient cells which do not contain the vector; the number of copies of the vector which are desired in a particular host; and whether it is desirable to be able to "shuttle" the vector between host cells of different species.

Preferred prokaryotic vectors known in the art include plasmids such as those capable of replication in E. coli (such as, for example, pBR322, ColE1, pSC101, pACYC 184, pi VX). Such plasmids are, for example, disclosed by Maniatis, T., et al. (Molecular Cloning, A Laboratory Manual, Second Edition, Cold Spring Harbor Press, Cold Spring Harbor, N.Y. (1989); Ausubel, infra. Bacillus plasmids include pC194, pC221, pT127, etc. Such plasmids are disclosed by Gryczan, T. (In: The Molecular Biology of the Bacilli, Academic Press, NY (1982), pp. 307-329). Suitable Streptomyces plasmids include pIJ101 (Kendall, K. J., et al., J. Bacteriol. 169: 4177-444183 (1987)), and streptomyces bacteriophages such as phi C31 (Chater, K. F., et al., In: Sixth International Symposium on Actinomycetales Biology, Akademiai Kaido, Budapest, Hungary (1986), pp. 4445-5444). Pseudomonas plasmids are reviewed by John, J. F., et al. (Rev. Infect. Dis. 8: 693-704 (1986)), and Izaki, K. (Jpn. J. Bacteriol. 33: 729-74442 (1978); and Ausubel et al, supra).

Alternatively, gene expression elements useful for the expression of cDNA encoding anti-TNF antibodies or peptides include, but are not limited to (a) viral transcription promoters and their enhancer elements, such as the SV40 early promoter (Okayama, et al., Mol. Cell. Biol. 3: 280 (1983)), Rous sarcoma virus LTR (Gorman, et al., Proc. Natl. Acad. Sci., USA 79: 6777 (1982)), and Moloney murine leukemia virus LTR (Grosschedl, et al., Cell 41: 885 (1985)); (b) splice regions and polyadenylation sites such as those derived from the SV40 late region (Okayama et al., infra); and (c) polyadenylation sites such as in SV40 (Okayama et al., infra).

Immunoglobulin cDNA genes can be expressed as described by Liu et al., infra, and Weidle et al., Gene 51: 21 (1987), using as expression elements the SV40 early promoter and its enhancer, the mouse immunoglobulin H chain promoter enhancers, SV40 late region mRNA splicing, rabbit beta -globin intervening sequence, immunoglobulin and rabbit beta -globin

polyadenylation sites, and SV40 polyadenylation elements. For immunoglobulin genes comprised of part cDNA, part genomic DNA (Whittle et al., Protein Engineering 1: 499 (1987)), the transcriptional promoter is human cytomegalovirus, the promoter enhancers are cytomegalovirus and mouse/human immunoglobulin, and mRNA splicing and polyadenylation regions are from the native chromosomal immunoglobulin sequences.

In one embodiment, for expression of cDNA genes in rodent cells, the transcriptional promoter is a viral LTR sequence, the transcriptional promoter enhancers are either or both the mouse immunoglobulin heavy chain enhancer and the viral LTR enhancer, the splice region contains an intron of greater than 31 bp, and the polyadenylation and transcription termination regions are derived from the native chromosomal sequence corresponding to the immunoglobulin chain being synthesized. In other embodiments, cDNA sequences encoding other proteins are combined with the above-recited expression elements to achieve expression of the proteins in mammalian cells.

Each fused gene is assembled in, or inserted into, an expression vector. Recipient cells capable of expressing the chimeric immunoglobulin chain gene product are then transfected singly with an anti-TNF peptide or chimeric H or chimeric L chain-encoding gene, or are co-transfected with a chimeric H and a chimeric L chain gene. The transfected recipient cells are cultured under conditions that permit expression of the incorporated genes and the expressed immunoglobulin chains or intact antibodies or fragments are recovered from the culture.

In one embodiment, the fused genes encoding the anti-TNF peptide or chimeric H and L chains, or portions thereof, are assembled in separate expression vectors that are then used to co-transfect a recipient cell.

Each vector can contain two selectable genes, a first selectable gene designed for selection in a bacterial system and a second selectable gene designed for selection in a eukaryotic system, wherein each vector has a different pair of genes. This strategy results in vectors which first direct the production, and permit amplification, of the fused genes in a bacterial system. The genes so produced and amplified in a bacterial host are subsequently used to co-transfect a eukaryotic cell, and allow selection of a co-transfected cell carrying the desired transfected genes.

Examples of selectable genes for use in a bacterial system are the gene that confers resistance to ampicillin and the gene that confers resistance to chloramphenicol. Preferred selectable genes for use in eukaryotic transfectants include the xanthine guanine phosphoribosyl transferase gene (designated gpt) and the phosphotransferase gene from Tn5 (designated neo).

Selection of cells expressing gpt is based on the fact that the enzyme encoded by this gene utilizes xanthine as a substrate for purine nucleotide synthesis, whereas the analogous endogenous enzyme cannot. In a medium containing (1) mycophenolic acid, which blocks the conversion of inosine monophosphate to xanthine monophosphate, and (2) xanthine, only cells expressing the gpt gene can survive. The product of the neo blocks the inhibition of protein synthesis by the antibiotic G418 and other antibiotics of the neomycin class.

The two selection procedures can be used simultaneously or sequentially to select for the expression of immunoglobulin chain genes introduced on two different DNA vectors into a eukaryotic cell. It is not necessary to include different selectable markers for eukaryotic cells; an H and an L chain vector, each containing the same selectable marker can be co-transfected. After selection of the appropriately resistant cells, the majority of the clones will contain integrated copies of both H and L chain vectors and/or anti-TNF peptides.

Alternatively, the fused genes encoding the chimeric H and L chains can be assembled on the

same expression vector.

For transfection of the expression vectors and production of the chimeric antibody, the preferred recipient cell line is a myeloma cell. Myeloma cells can synthesize, assemble and secrete immunoglobulins encoded by transfected immunoglobulin genes and possess the mechanism for glycosylation of the immunoglobulin. A particularly preferred recipient cell is the recombinant Ig-producing myeloma cell SP2/0 (ATCC #CRL 8287). SP2/0 cells produce only immunoglobulin encoded by the transfected genes. Myeloma cells can be grown in culture or in the peritoneal cavity of a mouse, where secreted immunoglobulin can be obtained from ascites fluid. Other suitable recipient cells include lymphoid cells such as B lymphocytes of human or non-human origin, hybridoma cells of human or non-human origin, or interspecies heterohybridoma cells.

The expression vector carrying a chimeric antibody construct or anti-TNF peptide of the present invention can be introduced into an appropriate host cell by any of a variety of suitable means, including such biochemical means as transformation, transfection, conjugation, protoplast fusion, calcium phosphate-precipitation, and application with polycations such as diethylaminoethyl (DEAE) dextran, and such mechanical means as electroporation, direct microinjection, and microprojectile bombardment (Johnston et al., Science 240: 1538 (1988)). A preferred way of introducing DNA into lymphoid cells is by electroporation (Potter et al., Proc. Natl. Acad. Sci. USA 81: 7161 (1984); Yoshikawa, et al., Jpn. J. Cancer Res. 77: 1122-1133). In this procedure, recipient cells are subjected to an electric pulse in the presence of the DNA to be incorporated. Typically, after transfection, cells are allowed to recover in complete medium for about 24 hours, and are then seeded in 96-well culture plates in the presence of the selective medium. G418 selection is performed using about 0.4 to 0.8 mg/ml G418. Mycophenolic acid selection utilizes about 6 mu /ml plus about 0.25 mg/ml xanthine. The electroporation technique is expected to yield transfection frequencies of about 10< - 5 > to about 10< - 4 > for Sp2/0 cells. In the protoplast fusion method, lysozyme is used to strip cell walls from catarrhal harboring the recombinant plasmid containing the chimeric antibody gene. The resulting spheroplasts are fused with myeloma cells with polyethylene glycol.

The immunoglobulin genes of the present invention can also be expressed in nonlymphoid mammalian cells or in other eukaryotic cells, such as yeast, or in prokaryotic cells, in particular bacteria.

Yeast provides substantial advantages over bacteria for the production of immunoglobulin H and L chains. Yeasts carry out post-translational peptide modifications including glycosylation. A number of recombinant DNA strategies now exist which utilize strong promoter sequences and high copy number plasmids which can be used for production of the desired proteins in yeast. Yeast recognizes leader sequences of cloned mammalian gene products and secretes peptides bearing leader sequences (i.e., pre-peptides) (Hitzman, et al., 11th International Conference on Yeast, Genetics and Molecular Biology, Montpelier, France, Sep. 13-17, 1982).

Yeast gene expression systems can be routinely evaluated for the levels of production, secretion and the stability of anti-TNF peptides, antibody and assembled murine and chimeric antibodies, fragments and regions thereof. Any of a series of yeast gene expression systems incorporating promoter and termination elements from the actively expressed genes coding for glycolytic enzymes produced in large quantities when yeasts are grown in media rich in glucose can be utilized. Known glycolytic genes can also provide very efficient transcription control signals. For example, the promoter and terminator signals of the phosphoglycerate kinase (PGK) gene can be utilized. A number of approaches can be taken for evaluating optimal expression plasmids for the expression of cloned immunoglobulin cDNAs in yeast (see Glover, ed., DNA Cloning, Vol. II, pp 45-66, IRL Press, 1985).

Bacterial strains can also be utilized as hosts for the production of antibody molecules or peptides described by this invention, E. coli K12 strains such as E. coli W3110 (ATCC 27325), and other enterobacteria such as Salmonella typhimurium or Serratia marcescens, and various Pseudomonas species can be used.

Plasmid vectors containing replicon and control sequences which are derived from species compatible with a host cell are used in connection with these bacterial hosts. The vector carries a replication site, as well as specific genes which are capable of providing phenotypic selection in transformed cells. A number of approaches can be taken for evaluating the expression plasmids for the production of murine and chimeric antibodies, fragments and regions or antibody chains encoded by the cloned immunoglobulin cDNAs in bacteria (see Glover, ed., DNA Cloning, Vol. I, IRL Press, 1985, Ausubel, infra, Sambrook, infra, Colligan, infra).

Preferred hosts are mammalian cells, grown in vitro or in vivo. Mammalian cells provide post-translational modifications to immunoglobulin protein molecules including leader peptide removal, folding and assembly of H and L chains, glycosylation of the antibody molecules, and secretion of functional antibody protein.

Mammalian cells which can be useful as hosts for the production of antibody proteins, in addition to the cells of lymphoid origin described above, include cells of fibroblast origin, such as Vero (ATCC CRL 81) or CHO-K1 (ATCC CRL 61).

Many vector systems are available for the expression of cloned anti TNF peptides H and L chain genes in mammalian cells (see Glover, ed., DNA Cloning, Vol. II, pp 143-238, IRL Press, 1985). Different approaches can be followed to obtain complete H2L2 antibodies. As discussed above, it is possible to co-express H and L chains in the same cells to achieve intracellular association and linkage of H and L chains into complete tetrameric H2L2 antibodies and/or anti-TNF peptides. The co-expression can occur by using either the same or different plasmids in the same host. Genes for both H and L chains and/or anti-TNF peptides can be placed into the same plasmid, which is then transfected into cells, thereby selecting directly for cells that express both chains. Alternatively, cells can be transfected first with a plasmid encoding one chain, for example the L chain, followed by transfection of the resulting cell line with an H chain plasmid containing a second selectable marker. Cell lines producing anti-TNF peptides and/or H2L2 molecules via either route could be transfected with plasmids encoding additional copies of peptides, H, L, or H plus L chains in conjunction with additional selectable markers to generate cell lines with enhanced properties, such as higher production of assembled H2L2 antibody molecules or enhanced stability of the transfected cell lines.

Anti-idiotype Abs. In addition to monoclonal or chimeric anti-TNF antibodies, the present invention is also directed to an anti-idiotypic (anti-Id) antibody specific for the anti-TNF antibody of the invention. An anti-Id antibody is an antibody which recognizes unique determinants generally associated with the antigen-binding region of another antibody. The antibody specific for TNF is termed the idiotypic or Id antibody. The anti-Id can be prepared by immunizing an animal of the same species and genetic type (e.g. mouse strain) as the source of the Id antibody with the Id antibody or the antigen-binding region thereof. The immunized animal will recognize and respond to the idiotypic determinants of the immunizing antibody and produce an anti-Id antibody. The anti-Id antibody can also be used as an "immunogen" to induce an immune response in yet another animal, producing a so-called anti-anti-Id antibody. The anti-anti-Id can be epitopically identical to the original antibody which induced the anti-Id. Thus, by using antibodies to the idiotypic determinants of a mAb, it is possible to identify other clones expressing antibodies of identical specificity.

Accordingly, mAbs generated against TNF according to the present invention can be used to induce anti-Id antibodies in suitable animals, such as BALB/c mice. Spleen cells from such

immunized mice can be used to produce anti-Id hybridomas secreting anti-Id mAbs. Further, the anti-Id mAbs can be coupled to a carrier such as keyhole limpet hemocyanin (KLH) and used to immunize additional BALB/c mice. Sera from these mice will contain anti-anti-Id antibodies that have the binding properties of the original mAb specific for a TNF epitope.

Screening Methods for determining tissue necrosis factor neutralizing and/or inhibiting activity are also provided in the present invention. In the context of the present invention, TNF neutralizing activity or TNF inhibiting activity refers to the ability of a TNF neutralizing compound to block at least one biological activity of TNF, such as preventing TNF from binding to a TNF receptor, blocking production of TNF by intracellular processing, such as transcription, translation or post-translational modification, expression on the cell surface, secretion or assembly of the bioactive trimer of TNF. Additionally, TNF neutralizing compounds can act by inducing regulation of metabolic pathways such as those involving the up or down regulation of TNF production. Alternatively TNF neutralizing compounds can modulate cellular sensitivity to TNF by decreasing such sensitivity. TNF neutralizing compounds can be selected from the group consisting of antibodies, or fragments or portions thereof, peptides, peptido mimetic compounds or organo mimetic compounds that neutralizes TNF activity in vitro, in situ or in vivo is considered a TNF neutralizing compound if used according to the present invention. Screening methods which can be used to determine TNF neutralizing activity of a TNF neutralizing compound can include in vitro or in vivo assays. Such in vitro assays can include a TNF cytotoxicity assay, such as a radioimmuno assay which determine a decrease in cell death by contact with TNF, such as chimpanzee or human TNF in isolated or recombinant form, wherein the concurrent presence of a TNF neutralizing compound reduces the degree or rate of cell death. The cell death can be determined using ID50 values which represent the concentration of a TNF neutralizing compound which decreases the cell death rate by 50%. For example, MAb's A2 and cA2 are found to have ID50 about 17 mg/ml +/- 3 mg/ml, such as 14-20 mg/ml, or any range or value therein. Such a TNF cytotoxicity assay is presented in example II.

Alternatively or additionally, another in vitro assay which can be used to determine neutralizing activity of a TNF neutralizing compound is an assay which measures the neutralization of TNF induced procoagulant activity, such as presented in example XI.

Alternatively or additionally, TNF neutralizing activity of a TNF neutralizing compound can be measured by an assay for the neutralization of TNF induced IL-6 secretion, such as using cultured human umbilical vein endothelial cells (HUVEC), for example. Also presented in example 11.

Alternatively or additionally, in vivo testing of TNF neutralizing activity of TNF neutralizing compounds can be tested using survival of mouse given lethal doses of Rh TNF with controlled and varied concentrations of a TNF neutralizing compound, such as TNF antibodies. Preferably galactosamine sensitive mice are used. For example, using a chimeric human anti-TNF antibody as a TNF neutralizing compound, a concentration of 0.4 milligrams per kilogram TNF antibody resulted in a 70-100% increase in survival and a 2.0 mg/kg dose of TNF antibody resulted in a 90-100% increase in survival rate using such an assay, for example, as presented in example 12.

Additionally, after TNF neutralizing compounds are tested for safety in animal models such as chimpanzees, for example as presented in Example XVII, TNF neutralizing compounds can be used to treat various TNF related pathologies, as described herein, and as presented in Examples XVIII-XXII.

Accordingly, any suitable TNF neutralizing compound can be used in methods according to the present invention. Examples of such TNF neutralizing compound can be selected from the group consisting of antibodies or portions thereof specific to neutralizing epitopes of TNF, p55 receptors, p75 receptors, or complexes thereof, portions of TNF receptors which bind TNF,

peptides which bind TNF, any peptido mimetic drugs which bind TNF and any organo mimetic drugs that block TNF.

Such TNF neutralizing compounds can be determined by routine experimentation based on the teachings and guidance presented herein, by those skilled in the relevant arts.

Structural Analogs of Anti-TNF Antibodies and Anti-TNF Peptides

Structural analogs of anti-TNF Abs and peptides of the present invention are provided by known method steps based on the teaching and guidance presented herein.

Knowledge of the three-dimensional structures of proteins is crucial in understanding how they function. The three-dimensional structures of more than 400 proteins are currently available in protein structure databases (in contrast to around 15,000 known protein sequences in sequence databases). Analysis of these structures shows that they fall into recognizable classes of motifs. It is thus possible to model a three-dimensional structure of a protein based on the proteins homology to a related protein of known structure. Many examples are known where two proteins that have relatively low sequence homology, can have very similar three dimensional structures or motifs.

In recent years it has become possible to determine the three dimensional structures of proteins of up to about 15 kDa by nuclear magnetic resonance (NMR). The technique only requires a concentrated solution of pure protein. No crystals or isomorphous derivatives are needed. The structures of a number proteins have been determined by this method. The details of NMR structure determination are well-known in the art (See, e.g., Wuthrich, NMR of Proteins and Nucleic Acids, Wiley, N.Y., 1986; Wuthrich, K. Science 243: 45-50 (1989); Clore et al., Crit. Rev. Bioch. Molec. Biol. 24: 479-564 (1989); Cooke et al. Bioassays 8: 52-56 (1988), which references are hereby incorporated herein by reference).

In applying this approach, a variety of <1> H NMR 2D data sets are collected for anti-TNF Abs and/or anti-TNF peptides of the present invention. These are of two main types. One type, COSY (Correlated Spectroscopy) identifies proton resonances that are linked by chemical bonds. These spectra provide information on protons that are linked by three or less covalent bonds. NOESY (nuclear Overhauser enhancement spectroscopy) identifies protons which are close in space (less than 0.5 nm). Following assignment of the complete spin system, the secondary structure is defined by NOESY. Cross peaks (nuclear Overhauser effects or NOE's) are found between residues that are adjacent in the primary sequence of the peptide and can be seen for protons less than 0.5 nm apart. The data gathered from sequential NOE's combined with amide proton coupling constants and NOE's from non-adjacent amino acids, that are adjacent to the secondary structure, are used to characterize the secondary structure of the polypeptides. Aside from predicting secondary structure, NOE's indicate the distance that protons are in space in both the primary amino acid sequence and the secondary structures. Tertiary structure predictions are determined, after all the data are considered, by a "best fit" extrapolation.

Types of amino acid are first identified using through-bond connectivities. The second step is to assign specific amino acids using through-space connectivities to neighboring residues, together with the known amino acid sequence. Structural information is then tabulated and is of three main kinds: The NOE identifies pairs of protons which are close in space, coupling constants give information on dihedral angles and slowly exchanging amide protons give information on the position of hydrogen bonds. The restraints are used to compute the structure using a distance geometry type of calculation followed by refinement using restrained molecular dynamics. The output of these computer programs is a family of structures which are compatible with the experimental data (i.e. the set of pairwise < 0.5 nm distance restraints). The better that the structure is defined by the data, the better the family of structures can be superimposed, (i.e., the better the resolution of the structure). In the

better defined structures using NMR, the position of much of backbone (i.e. the amide, C alpha and carbonyl atoms) and the side chains of those amino acids that lie buried in the core of the molecule can be defined as clearly as in structures obtained by crystallography. The side chains of amino acid residues exposed on the surface are frequently less well defined, however. This probably reflects the fact that these surface residues are more mobile and can have no fixed position. (In a crystal structure this might be seen as diffuse electron density).

Thus, according to the present invention, use of NMR spectroscopic data is combined with computer modeling to arrive structural analogs of at least portions of anti-TNF Abs and peptides based on a structural understanding of the topography. Using this information, one of ordinary skill in the art will know how to achieve structural analogs of anti-TNF Abs and/or peptides, such as by rationally-based amino acid substitutions allowing the production of peptides in which the TNF binding affinity is modulated in accordance with the requirements of the expected therapeutic or diagnostic use of the molecule, preferably, the achievement of greater specificity for TNF binding.

Alternatively, compounds having the structural and chemical features suitable as anti-TNF therapeutics and diagnostics provide structural analogs with selective TNF affinity. Molecular modeling studies of TNF binding compounds, such as TNF receptors, anti-TNF antibodies, or other TNF binding molecules, using a program such as MACROMODEL Registered TM , INSIGHT Registered TM , and DISCOVER Registered TM provide such spatial requirements and orientation of the anti-TNF Abs and/or peptides according to the present invention. Such structural analogs of the present invention thus provide selective qualitative and quantitative anti-TNF activity in vitro, in situ and/or in vivo.

Therapeutic Methods for Treating TNF-Related Pathologies The anti-TNF peptides, antibodies, fragments and/or derivatives of the present invention are useful for treating a subject having a pathology or condition associated with abnormal levels of a substance reactive with an anti-TNF antibody, in particular TNF, such as TNF alpha or TNF beta , in excess of, or less than, levels present in a normal healthy subject, where such excess or diminished levels occur in a systemic, localized or particular tissue type or location in the body. Such tissue types can include, but are not limited to, blood, lymph, CNS, liver, kidney, spleen, heart muscle or blood vessels, brain or spinal cord white matter or grey matter, cartilage, ligaments, tendons, lung, pancreas, ovary, testes, prostrate. Increased or decreased TNF concentrations relative to normal levels can also be localized to specific regions or cells in the body, such as joints, nerve blood vessel junctions, bones, specific tendons or ligaments, or sites of infection, such as bacterial or viral infections.

TNF related pathologies include, but are not limited to, the following:

(A) acute and chronic immune and autoimmune pathologies, such as systemic lupus erythematosus (SLE) rheumatoid arthritis, thyroidosis, graft versus host disease, scleroderma, diabetes mellitus, Graves' disease, and the like;

(B) infections, including, but not limited to, sepsis syndrome, cachexia, circulatory collapse and shock resulting from acute or chronic bacterial infection, acute and chronic parasitic and/or infectious diseases, bacterial, viral or fungal, such as a HIV, AIDS (including symptoms of cachexia, autoimmune disorders, AIDS dementia complex and infections);

(C) inflammatory diseases, such as chronic inflammatory pathologies and vacsular inflammatory pathologies, including chronic inflammatory pathologies such as sarcoidosis, chronic inflammatory bowel disease, ulcerative colitis, and Crohn's pathology and vascular inflammatory pathologies, such as, but not limited to, disseminated intravascular coagulation, atherosclerosis, and Kawasaki's pathology:

(D) neurodegenerative diseases, including, but are not limited to,

demyelinating diseases, such as multiple sclerosis and acute transverse myelitis;

extrapyramidal and cerebellar disorders' such as lesions of the corticospinal system;

disorders of the basal ganglia or cerebellar disorders;

hyperkinetic movement disorders such as Huntington's Chorea and senile chorea;

drug-induced movement disorders, such as those induced by drugs which block CNS dopamine receptors;

hypokinetic movement disorders, such as Parkinson's disease;

Progressive supranucleo palsy;

Cerebellar and Spinocerebellar Disorders, such as astructural lesions of the cerebellum;

spinocerebellar degenerations (spinal ataxia, Friedreich's ataxia, cerebellar cortical degenerations, multiple systems degenerations (Mencel, Dejerine-Thomas, Shi-Drager, and Machado-Joseph); and systemic disorders (Refsum's disease, abetalipoprotemia, ataxia, telangiectasia, and mitochondrial multi.system disorder);

demyelinating core disorders, such as multiple sclerosis, acute transverse myelitis;

disorders of the motor unit, such as neurogenic muscular atrophies (anterior horn cell degeneration, such as amyotrophic lateral sclerosis, infantile spinal muscular atrophy and juvenile spinal muscular atrophy); Alzheimer's disease; Down's Syndrome in middle age; Diffuse Lewy body disease; Senile Dementia of Lewy body type; Wernicke-Korsakoff syndrome; chronic alcoholism; Creutzfeldt-Jakob disease; Subacute sclerosing panencephalitis, Hallerrorden-Spatz disease; and Dementia pugilistica, or any subset thereof;

(E) malignant pathologies involving TNF-secreting tumors or other malignancies involving TNF, such as, but not limited to leukemias (acute, chronic myelocytic, chronic lymphocytic and/or myelodyspastic syndrome); lymphomas (Hodgkin's and non-Hodgkin's lymphomas, such as malignant lymphomas (Burkitt's lymphoma or Mycosis fungoides)); and

(F) alcohol-induced hepatitis.

See, e.g., Berkow et al, eds., The Merck Manual, 16th edition, chapter 11, pp 1380-1529, Merck and Co., Rahway, N.J., 1992, which reference, and references cited therein, are entirely incorporated herein by reference.

Such treatment comprises parenterally administering a single or multiple doses of the antibody, fragment or derivative. Preferred for human pharmaceutical use are high affinity potent hTNF alpha -inhibiting and/or neutralizing murine and chimeric antibodies, fragments and regions of this invention.

Anti-TNF peptides or MAbs of the present invention can be administered by any means that enables the active agent to reach the agent's site of action in the body of a mammal. In the case of the antibodies of this invention, the primary focus is the ability to reach and bind with TNF released by monocytes and macrophages or other TNF producing cells. Because proteins are subject to being digested when administered orally, parenteral administration, i.e., intravenous, subcutaneous, intramuscular, would ordinarily be used to optimize absorption.

Therapeutic Administration

Anti-TNF peptides and/or MAbs of the present invention can be administered either as individual therapeutic agents or in combination with other therapeutic agents. They can be administered alone, but are generally administered with a pharmaceutical carrier selected on the basis of the chosen route of administration and standard pharmaceutical practice.

The dosage administered will, of course, vary depending upon known factors such as the pharmacodynamic characteristics of the particular agent, and its mode and route of administration; age, health, and weight of the recipient; nature and extent of symptoms, kind of concurrent treatment, frequency of treatment, and the effect desired. Usually a daily dosage of active ingredient can be about 0.01 to 100 milligrams per kilogram of body weight. Ordinarily 1.0 to 5, and preferably 1 to 10 milligrams per kilogram per day given in divided doses 1 to 6 times a day or in sustained release form is effective to obtain desired results.

As a non-limiting example, treatment of TNF-related pathologies humans or animals can be provided as a daily dosage of anti-TNF peptides, monoclonal chimeric and/or murine antibodies of the present invention 0.1 to 100 mg/kg, such as 0.5, 0.9, 1.0, 1.1, 1.5, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 40, 45, 50, 60, 70, 80, 90 or 100 mg/kg, per day, on at least one of day 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, or 40, or alternatively, at least one of week 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20, or any combination thereof, using single or divided doses of every 24, 12, 8, 6, 4, or 2 hours, or any combination thereof.

Since circulating concentrations of TNF tend to be extremely low, in the range of about 10 pg/ml in non-septic individuals, and reaching about 50 pg/ml in septic patients and above 100 pg/ml in the sepsis syndrome (Hammerle, A. F. et al., 1989, infra) or can be only be detectable at sites of TNF-mediated pathology, it is preferred to use high affinity and/or potent in vivo TNF-inhibiting and/or neutralizing antibodies, fragments or regions thereof, for both TNF immunoassays and therapy of TNF-mediated pathology. Such antibodies, fragments, or regions, will preferably have an affinity for hTNF alpha , expressed as Ka, of at least $10^8 M^{-1}$, more preferably, at least $10^9 M^{-1}$, such as 10^8 -$10^{10} M^{-1}$, $5 \times 10^8 M^{-1}$, $8 \times 10^8 M^{-1}$, $2 \times 10^9 M^{-1}$, $4 \times 10^9 M^{-1}$, $6 \times 10^9 M^{-1}$, $8 \times 10^9 M^{-1}$, or any range or value therein.

Preferred for human therapeutic use are high affinity murine and chimeric antibodies, and fragments, regions and derivatives having potent in vivo TNF alpha -inhibiting and/or neutralizing activity, according to the present invention, that block TNF-induced IL-6 secretion. Also preferred for human therapeutic uses are such high affinity murine and chimeric anti-TNF alpha antibodies, and fragments, regions and derivatives thereof, that block TNF-induced procoagulant activity, including blocking of TNF-induced expression of cell, adhesion molecules such as ELAM-1 and ICAM-1 and blocking of TNF mitogenic activity, in vivo, in situ, and in vitro.

Dosage forms (composition) suitable for internal administration generally contain from about 0.1 milligram to about 500 milligrams of active ingredient per unit. In these pharmaceutical compositions the active ingredient will ordinarily be present in an amount of about 0.5-95% by weight based on the total weight of the composition.

For parenteral administration, anti-TNF peptides or antibodies can be formulated as a solution, suspension, emulsion or lyophilized powder in association with a pharmaceutically acceptable parenteral vehicle. Examples of such vehicles are water, saline, Ringer's solution, dextrose solution, and 5% human serum albumin. Liposomes and nonaqueous vehicles such as fixed oils can also be used. The vehicle or lyophilized powder can contain additives that

maintain isotonicity (e.g., sodium chloride, mannitol) and chemical stability (e.g., buffers and preservatives). The formulation is sterilized by commonly used techniques.

Suitable pharmaceutical carriers are described in the most recent edition of Remington's Pharmaceutical Sciences, A. Osol, a standard reference text in this field of art.

For example, a parenteral composition suitable for administration by injection is prepared by dissolving 1.5% by weight of active ingredient in 0.9% sodium chloride solution.

Anti-TNF peptides and/or antibodies of this invention can be adapted for therapeutic efficacy by virtue of their ability to mediate antibody-dependent cellular cytotoxicity (ADCC) and/or complement-dependent cytotoxicity (CDC) against cells having TNF associated with their surface. For these activities, either an endogenous source or an exogenous source of effector cells (for ADCC) or complement components (for CDC) can be utilized. The murine and chimeric antibodies, fragments and regions of this invention, their fragments, and derivatives can be used therapeutically as immunoconjugates (see for review: Dillman, R. O., Ann. Int. Med. 111: 592-603 (1989)). Such peptides or Abs can be coupled to cytotoxic proteins, including, but not limited to ricin-A, Pseudomonas toxin and Diphtheria toxin. Toxins conjugated to antibodies or other ligands or peptides are well known in the art (see, for example, Olsnes, S. et al., Immunol. Today 10: 291-295 (1989)). Plant and bacterial toxins typically kill cells by disrupting the protein synthetic machinery.

Anti-TNF peptides and/or antibodies of this invention can be conjugated to additional types of therapeutic moieties including, but not limited to, radionuclides, therapeutic agents, cytotoxic agents and drugs. Examples of radionuclides which can be coupled to antibodies and delivered in vivo to sites of antigen include <212> Bi, <131> I, <186> Re, and <90> Y, which list is not intended to be exhaustive. The radionuclides exert their cytotoxic effect by locally irradiating the cells, leading to various intracellular lesions, as is known in the art of radiotherapy.

Cytotoxic drugs which can be conjugated to anti-TNF peptides and/or antibodies and subsequently used for in vivo therapy include, but are not limited to, daunorubicin, doxorubicin, methotrexate, and Mitomycin C. Cytotoxic drugs interfere with critical cellular processes including DNA, RNA, and protein synthesis. For a description of these classes of drugs which are well known in the art, and their mechanisms of action, see Goodman, et al., Goodman and Gilman's THE PHARMACOLOGICAL BASIS OF THERAPEUTICS, 8th Ed., Macmillan Publishing Co., 1990.

Anti-TNF peptides and/or antibodies of this invention can be advantageously utilized in combination with other monoclonal or murine and chimeric antibodies, fragments and regions, or with lymphokines or hemopoietic growth factors, etc., which serve to increase the number or activity of effector cells which interact with the antibodies.

Anti-TNF peptides and/or antibodies, fragments or derivatives of this invention can also be used in combination with TNF therapy to block undesired side effects of TNF. Recent approaches to cancer therapy have included direct administration of TNF to cancer patients or immunotherapy of cancer patients with lymphokine activated killer (LAK) cells (Rosenberg et al., New Eng. J. Med. 313: 1485-1492 (1985)) or tumor infiltrating lymphocytes (TIL) (Kurnick et al. (Clin. Immunol. Immunopath. 38: 367-380 (1986); Kradin et al., Cancer Immunol. Immunother. 24: 76-85 (1987); Kradin et al., Transplant. Proc. 20: 336-338 (1988)). Trials are currently underway using modified LAK cells or TIL which have been transfected with the TNF gene to produce large amounts of TNF. Such therapeutic approaches are likely to be associated with a number of undesired side effects caused by the pleiotropic actions of TNF as described herein and known in the related arts. According to the present invention, these side effects can be reduced by concurrent treatment of a subject receiving TNF or cells producing large amounts of TIL with the antibodies, fragments or

derivatives of the present invention. Effective doses are as described above. The dose level will require adjustment according to the dose of TNF or TNF-producing cells administered, in order to block side effects without blocking the main anti-tumor effect of TNF. One of ordinary skill in the art will know how to determine such doses without undue experimentation.

Treatment of Arthritis. In rheumatoid arthritis, the main presenting symptoms are pain, stiffness, swelling, and loss of function (Bennett J C. The etiology of rheumatoid arthritis. In Textbook of Rheumatology (Kelley W N, Harris E D, Ruddy S, Sledge C B, eds.) W B Saunders, Philadelphia pp 879-886, 1985). The muititude of drugs used in controlling such symptoms seems largely to reflect the fact that none is ideal. Although there have been many years of intense research into the biochemical, genetic, microbiological, and immunological aspects of rheumatoid arthritis, its pathogenesis is not completely understood, and none of the treatments clearly stop progression of joint destruction (Harris E D. Rheumatoid Arthritis: The clinical spectrum. In Textbook of Rheumatology (Kelley, et al., eds.) W B Saunders, Philadelphia pp 915-990, 1985).

TNF alpha is of major importance in the pathogenesis of rheumatoid arthritis. TNF alpha is present in rheumatoid arthritis joint tissues and synovial fluid at the protein and mRNA level (Buchan G, Barrett K, Turner M, Chantry D, Maini R N, and Feldmann M. Interleukin-1 and tumour necrosis factor mRNA expression in rheumatoid arthritis: prolonged production of IL-1 alpha . Clin. Exp. Immunol 73: 449-455, 1988), indicating local synthesis. However detecting TNF alpha in rheumatoid arthritis joints even in quantities sufficient for bioactivation does not necessarily indicate that it is important in the pathogenesis of rheumatoid arthritis, nor that it is a good candidate therapeutic target. In order to address these questions, the effects of anti-TNF antibody and peptides (rabbit or monoclonal) on rheumatoid joint cell cultures, and for comparison, osteoarthritic cell cultures, have been studied. IL-1 production was abolished, showing TNF alpha as a suitable therapeutic target for the therapy of rheumatoid arthritis, since anti-TNF alpha blocks both TNF and IL-1, the two cytokines known to be involved in cartilage and bone destruction (Brennan et al., Lancet 11: 244-247, 1989).

Subsequent studies in rheumatoid arthritis tissues have supported this hypothesis. Anti-TNF Abs abrogated the production of another proinflammatory cytokine, GM-CSF (Haworth et al., Eur. J. Immunol. 21: 2575-2579, 1991). This observation has been independently confirmed (Alvaro-Gracia et al., 1991). It has also been demonstrated that anti-TNF diminishes cell adhesion and HLA class II expression in rheumatoid arthritis joint cell cultures.

Diagnostic Methods

The present invention also provides the above anti-TNF peptides and antibodies, detectably labeled, as described below, for use in diagnostic methods for detecting TNF alpha in patients known to be or suspected of having a TNF alpha -mediated condition.

Anti-TNF peptides and/or antibodies of the present invention are useful for immunoassays which detect or quantitate TNF, or anti-TNF antibodies, in a sample. An immunoassay for TNF typically comprises incubating a biological sample in the presence of a detectably labeled high affinity anti-TNF peptide and/or antibody of the present invention capable of selectively binding to TNF, and detecting the labeled peptide or antibody which is bound in a sample. Various clinical assay procedures are well known in the art, e.g, as described in Immunoassays for the 80's, A. Voller et al., eds., University Park, 1981.

Thus, an anti-TNF peptide or antibody, can be added to nitrocellulose, or other solid support which is capable of immobilizing cells, cell particles or soluble proteins. The support can then be washed with suitable buffers followed by treatment with the detectably labeled TNF-specific peptide or antibody. The solid phase support can then be washed with the buffer a

second time to remove unbound peptide or antibody. The amount of bound label on the solid support can then be detected by known method steps.

By "solid phase support" or "carrier" is intended any support capable of binding peptide, antigen or antibody. Well-known supports or carriers, include glass, polystyrene, polypropylene, polyethylene, dextran, nylon, amylases, natural and modified celluloses, polyacrylamides, agaroses, and magnetite. The nature of the carrier can be either soluble to some extent or insoluble for the purposes of the present invention. The support material can have virtually any possible structural configuration so long as the coupled molecule is capable of binding to TNF or an anti-TNF antibody. Thus, the support configuration can be spherical, as in a bead, or cylindrical, as in the inside surface of a test tube, or the external surface of a rod. Alternatively, the surface can be flat such as a sheet, culture dish, test strip, etc. Preferred supports include polystyrene beads. Those skilled in the art will know many other suitable carriers for binding antibody, peptide or antigen, or can ascertain the same by routine experimentation.

Well known method steps can determine binding activity of a given lot of anti-TNF peptide and/or antibody. Those skilled in the art can determine operative and optimal assay conditions by routine experimentation.

Detectably labeling a TNF-specific peptide and/or antibody can be accomplished by linking to an enzyme for use in an enzyme immunoassay (EIA), or enzyme-linked immunosorbent assay (ELISA). The linked enzyme reacts with the exposed substrate to generate a chemical moiety which can be detected, for example, by spectrophotometric, fluorometric or by visual means. Enzymes which can be used to detectably label the TNF-specific antibodies of the present invention include, but are not limited to, malate dehydrogenase, staphylococcal nuclease, delta-5-steroid isomerase, yeast alcohol dehydrogenase, alpha-glycerophosphate dehydrogenase, triose phosphate isomerase, horseradish peroxidase, alkaline phosphatase, asparaginase, glucose oxidase, beta-galactosidase, ribonuclease, urease, catalase, glucose-6-phosphate dehydrogenase, glucoamylase and acetylcholinesterase.

By radioactively labeling the TNF-specific antibodies, it is possible to detect TNF through the use of a radioimmunoassay (RIA) (see, for example, Work, et al., Laboratory Techniques and Biochemistry in Molecular Biology, North Holland Publishing Company, N.Y. (1978). The radio-active isotope can be detected by such means as the use of a gamma counter or a scintillation counter or by autoradiography. Isotopes which are particularly useful for the purpose of the present invention are: <3> H, <125> I, <131> I, <35> S, <14> C, and, preferably, <125> I.

It is also possible to label the TNF-specific antibodies with a fluorescent compound. When the fluorescent labeled antibody is exposed to light of the proper wave length, its presence can then be detected due to fluorescence. Among the most commonly used fluorescent labelling compounds are fluorescein isothiocyanate, rhodamine, phycoerythrin, phycocyanin, allophycocyanin, o-phthaldehyde and fluorescamine.

The TNF-specific antibodies can also be detectably labeled using fluorescence-emitting metals such as <152> Eu, or others of the lanthanide series. These metals can be attached to the TNF-specific antibody using such metal chelating groups as diethylenetriaminepentaacetic acid (DTPA) or ethylenediamine-tetraacetic acid (EDTA).

The TNF-specific antibodies also can be detectably labeled by coupling to a chemiluminescent compound. The presence of the chemiluminescently labeled antibody is then determined by detecting the presence of luminescence that arises during the course of a chemical reaction. Examples of particularly useful chemiluminescent labeling compounds are luminol, isoluminol, theromatic acridinium ester, imidazole, acridinium salt and oxalate ester.

Likewise, a bioluminescent compound can be used to label the TNF-specific antibody, fragment or derivative of the present invention. Bioluminescence is a type of chemiluminescence found in biological systems in which a catalytic protein increases the efficiency of the chemiluminescent reaction. The presence of a bioluminescent protein is determined by detecting the presence of luminescence. Important bioluminescent compounds for purposes of labeling are luciferin, luciferase and aequorin.

Detection of the TNF-specific antibody, fragment or derivative can be accomplished by a scintillation counter, for example, if the detectable label is a radioactive gamma emitter, or by a fluorometer, for example, if the label is a fluorescent material. In the case of an enzyme label, the detection can be accomplished by colorometric methods which employ a substrate for the enzyme. Detection can also be accomplished by visual comparison of the extent of enzymatic reaction of a substrate in comparison with similarly prepared standards.

For the purposes of the present invention, the TNF which is detected by the above assays can be present in a biological sample. Any sample containing TNF can be used. Preferably, the sample is a biological fluid such as, for example, blood, serum, lymph, urine, inflammatory exudate, cerebrospinal fluid, amniotic fluid, a tissue extract or homogenate, and the like. However, the invention is not limited to assays using only these samples, it being possible for one of ordinary skill in the art to determine suitable conditions which allow the use of other samples.

In situ detection can be accomplished by removing a histological specimen from a patient, and providing the combination of labeled antibodies of the present invention to such a specimen. The antibody (or fragment) is preferably provided by applying or by overlaying the labeled antibody (or fragment) to a biological sample. Through the use of such a procedure, it is possible to determine not only the presence of TNF but also the distribution of TNF in the examined tissue. Using the present invention, those of ordinary skill will readily perceive that any of a wide variety of histological methods (such as staining procedures) can be modified in order to achieve such in situ detection.

The antibody, fragment or derivative of the present invention can be adapted for utilization in an immunometric assay, also known as a "two-site" or "sandwich" assay. In a typical immunometric assay, a quantity of unlabeled antibody (or fragment of antibody) is bound to a solid support that is insoluble in the fluid being tested and a quantity of detectably labeled soluble antibody is added to permit detection and/or quantitation of the ternary complex formed between solid-phase antibody, antigen, and labeled antibody.

Typical, and preferred, immunometric assays include "forward" assays in which the antibody bound to the solid phase is first contacted with the sample being tested to extract the TNF from the sample by formation of a binary solid phase antibody-TNF complex. After a suitable incubation period, the solid support is washed to remove the residue of the fluid sample, including unreacted TNF, if any, and then contacted with the solution containing a known quantity of labeled antibody (which functions as a "reporter molecule"). After a second incubation period to permit the labeled antibody to complex with the TNF bound to the solid support through the unlabeled antibody, the solid support is washed a second time to remove the unreacted labeled antibody. This type of forward sandwich assay can be a simple "yes/no" assay to determine whether TNF is present or can be made quantitative by comparing the measure of labeled antibody with that obtained for a standard sample containing known quantities of TNF. Such "two-site" or "sandwich" assays are described by Wide (Radioimmune Assay Method, Kirkham, ed., Livingstone, Edinburgh, 1970, pp. 199-206).

Other type of "sandwich" assays, which can also be useful with TNF, are the so-called "simultaneous" and "reverse" assays. A simultaneous assay involves a single incubation step wherein the antibody bound to the solid support and labeled antibody are both added to the

sample being tested at the same time. After the incubation is completed, the solid support is washed to remove the residue of fluid sample and uncomplexed labeled antibody. The presence of labeled antibody associated with the solid support is then determined as it would be in a conventional "forward" sandwich assay.

In the "reverse" assay, stepwise addition first of a solution of labeled antibody to the fluid sample followed by the addition of unlabeled antibody bound to a solid support after a suitable incubation period, is utilized. After a second incubation, the solid phase is washed in conventional fashion to free it of the residue of the sample being tested and the solution of unreacted labeled antibody. The determination of labeled antibody associated with a solid support is then determined as in the "simultaneous" and "forward" assays. In one embodiment, a combination of antibodies of the present invention specific for separate epitopes can be used to construct a sensitive three-site immunoradiometric assay.

TNF Removal From Solutions

The murine and chimeric antibodies, fragments and regions, fragments, or derivatives of this invention, attached to a solid support, can be used to remove TNF from fluids or tissue or cell extracts. In a preferred embodiment, they are used to remove TNF from blood or blood plasma products. In another preferred embodiment, the murine and chimeric antibodies, fragments and regions are advantageously used in extracorporeal immunoadsorbent devices, which are known in the art (see, for example, Seminars in Hematology, 26 (2 Suppl. 1) (1989)). Patient blood or other body fluid is exposed to the attached antibody, resulting in partial or complete removal of circulating TNF (free or in immune complexes), following which the fluid is returned to the body. This immunoadsorption can be implemented in a continuous flow arrangement, with or without interposing a cell centrifugation step. See, for example, Terman, et al., J. Immunol. 117: 1971-1975 (1976).

Having now generally described the invention, the same will be further understood by reference to certain specific examples which are included herein for purposes of illustration only and are not intended to be limiting unless otherwise specified.

EXAMPLE I

Production a Mouse Anti-Human TNF mAb

To facilitate clinical study of TNF mAb a high-affinity potent inhibiting and/or neutralizing mouse anti-human TNF IgG1 mAb designated A2 was produced.

Female BALB/c mice, 10 weeks old, were obtained from the Jackson Laboratory (Bar Harbor, Me.). Forty mu g of purified E. coli-derived recombinant human TNF (rhTNF) emulsified with an equal volume of complete Freund's adjuvant (obtained from Difco Laboratories) in 0.4 ml was injected subcutaneously and intraperitoneally (i.p.) into a mouse. One week later, an injection of 5 mu g of rhTNF in incomplete Freund's adjuvant was given i.p. followed by four consecutive i.p. injections of 10 mu g of TNF without adjuvant. Eight weeks after the last injection, the mouse was boosted i.p. with 10 mu g of TNF.

Four days later, the mouse was sacrificed, the spleen was obtained and a spleen cell suspension was prepared. Spleen cells were fused with cells of the nonsecreting hybridoma, Sp2/0 (ATCC CRL1581), at a 4:1 ratio of spleen cells to Sp2/0 cells, in the presence of 0.3 ml of 30% polyethylene glycol, PEG 1450. After incubation at 37o C. for 6 hours, the fused cells were distributed in 0.2 ml aliquots into 96-well plates at concentrations of 2 x 10<4 > SP2/0 cells per well. Feeder cells, in the form of 5 x 10<4 > normal BALB/c spleen cells, were added to each well.

The growth medium used consisted of RPMI-1640 medium, 10% heat-inactivated fetal bovine serum (FBS) (HYCLONE), 0.1 mM minimum essential medium (MEM) nonessential amino acids, 1 mM sodium pyruvate, 2 mM L-glutamine, 100 U/ml penicillin, 100 mu g/ml streptomycin (GIBCO Laboratories) and, for selection, hypoxanthine-aminopterin-thymidine (HAT) (Boehringer Mannheim). A solid-phase radioimmunoassay (RIA) was employed for screening supernatants for the presence of mAbs specific for rhTNF alpha . This assay is described in Example II, below. The background binding in this assay was about 500 cpm. A supernatant was considered positive if it yielded binding of 2000 cpm or higher.

Of 322 supernatants screened, 25 were positive by RIA. Of these 25, the one with the highest binding (4800 cpm) was designated A2. Positive wells were subcloned at limiting dilution on mouse feeder cells. Upon further analysis of the supernatants in neutralization assays, A2 was found to be the only positive clone showing potent inhibiting and/or neutralizing activity. Thus, the hybridoma line A2 was selected. This line was maintained in RPMI-1640 medium with 10% FBS (GIBCO), 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 2 mM L-glutamine, 100 U/ml penicillin and 100 mu g/ml streptomycin.

Alternatively, anti-TNF antibodies which inhibit TNF biological activity can be screened by binding to peptide including at least 5 amino acids of residues 87-108 or both residues 59-80 and 87-108 of TNF (of SEQ ID NO:1) or combinations of peptides contained therein, which are used in place of the rTNF protein, as described above.

EXAMPLE II

Characterization of an Anti-TNF Antibody of the Present Invention

Radioimmunoassays

E. coli-derived rhTNF was diluted to 1 mu g/ml in BCB buffer, pH 9.6, and 0.1 ml of the solution was added to each assay well. After incubation at 4o C. overnight, the wells were washed briefly with BCB, then sealed with 1% bovine incubated with 40 pg/ml of natural (GENZYME, Boston, Mass.) or recombinant (SUNTORY, Osaka, Japan) human TNF alpha with varying concentrations of mAb A2 in the presence of 20 mu g/ml cycloheximide at 39o C. Overnight. Controls included medium alone or medium + TNF in each well. Cell death was measured by staining with naphthol blue-black, and the results read spectrophotometrically at 630 nm. Absorbance at this wave length correlates with the number of live cells present.

It was found that A2 inhibited or neutralized the cytotoxic effect of both natural and rhTNF in a dose-dependent manner (FIG. 3).

In another experiment, the specificity of this inhibiting and/or neutralizing activity was tested. A673/6 cells were seeded at 3 x 10<4 > cells/well 20 hr before the TNF bioassay. Two-fold serial dilutions of rhTNF, E. coli-derived recombinant human lymphotoxin (TNF beta), and E. coli-derived recombinant murine TNF were prepared. The A2 hybridoma supernatant was added to an equal volume of the diluted TNF preparations, and the mixtures were incubated at room temperature for 30 min. Aliquots of 0.1 ml were transferred to the wells containing A673/6 cells, 20 mu g/ml of cycloheximide was added, and the cells were incubated at 39o C. overnight. The cells were then fixed and stained for evaluation of cytotoxicity. The results indicate that mAb A2 specifically inhibited or neutralized the cytotoxicity of rhTNF alpha , whereas it had no effect on human lymphotoxin (TNF beta) (FIG. 4) or murine TNF (FIG. 5).

Experiments were next performed to analyze the cross-reactivity of mAb A2 with TNF derived

from non-human primates. Monocytes isolated from B514 (baboon), J91 (cynomolgus) and RH383 (rhesus) blood by Ficoll gradient centrifugation and adherence, were incubated at 1 x 10<5 > cells/well in RPM1 1640 medium with 5% FBS and 2 mu g/ml of E. coli LPS for 3 or 16 hr at 37o C. to induce TNF production. Supernatants from duplicate wells were pooled and stored at 4o C. for less than 20 hr until the TNF bioassay was performed, as described above, using A673/6 cells. Two-fold dilutions of the culture supernatants were mixed with either medium or purified mAb A2 at a final concentration of 1 mu g/ml, incubated at room temperature for 30 min and aliquots transferred to the indicator cells. The results showed that mAb A2 failed to significantly inhibit or neutralize the cytotoxic activity of TNF produced by baboon, cynomolgus and rhesus monkey monocytes.

A further experiment was conducted with chimpanzee TNF. Monocytes isolated from CH563 (chimpanzee) blood were incubated as described above to generate TNF-containing supernatants. The ability of 10 mu g/ml of mAb A2 to inhibit or neutralize the bioactivity of these supernatants was assayed as above. Human TNF was used as a positive control. Results, shown in FIG. 6, indicate that mAb A2 had potent inhibiting and/or neutralizing activity for chimpanzee TNF, similar to that for human TNF (FIG. 7).

The inhibiting and/or neutralizing activity of mAb A2 was compared with three other murine mAbs specific for human TNF, termed TNF-1, TNF-2 and TNF-3, and a control mAb. Two-fold serial dilutions of purified mAbs were mixed with rhTNF (40 pg/ml), incubated at room temperature for 30 min, and aliquots tested for TNF bioactivity as above. It was found that mAbs TNF-1, TNF-2 and TNF-3 each had a similar moderate degree of inhibiting and/or neutralizing activity. In contrast, mAb A2 had much more potent inhibiting and/or neutralizing activity.

EXAMPLE III

General Strategy for Cloning Antibody V and C Genes

The strategy for cloning the V regions for the H and L chain genes from the hybridoma A2, which secretes the anti-TNF antibody described above, was based upon the linkage in the genome between the V region and the corresponding J (joining) region for functionally rearranged (and expressed) Ig genes. J region DNA probes can be used to screen genomic libraries to isolate DNA linked to the J regions. Although DNA in the germline configuration (i.e., unrearranged) would also hybridize to J probes, this DNA would not be linked to a Ig V region sequence and can be identified by restriction enzyme analysis of the isolated clones.

The cloning utilized herein was to isolate V regions from rearranged H and L chain genes using J[H]and J[k]probes. These clones were tested to see if their sequences were expressed in the A2 hybridoma by Northern analysis. Those clones that contained expressed sequence were cloned into expression vectors containing human C regions and transfected into mouse myeloma cells to determine if an antibody was produced. The antibody from producing cells was then tested for binding specificity and functionally compared to the A2 murine antibody.

EXAMPLE IV

Construction of a L Chain Genomic Library

To isolate the L chain V region gene from the A2 hybridoma, a size-selected genomic library was constructed using the phage lambda vector charon 27. High molecular weight DNA was

isolated from A2 hybridoma cells and digested to completion with restriction endonuclease HindIII. The DNA was then fractionated on a 0.8% agarose gel and the DNA fragments of three different size ranges of approximately 3 kb, 4 kb and 6 kb were isolated from the gel by electroelution. The size ranges for library construction were chosen based upon the size of Hind III fragments that hybridized on a southern blot with the J[k]probe. After phenol/chloroform extraction and ethanol precipitation, the DNA fragments from each size class were ligated with lambda charon 27 arms and packaged ingo phage particles in vitro using Gigapack Gold from Stratagens (LaJolla, Calif.).

These libraries were screened directly at a density of approximately 20,000 plaques per 150 mm petri dish using a <32> P-labeled J[k]probe. The mouse L chain J[k]probe was a 2.7 kb HindIII fragment containing all five J[k]segments. The probe was labeled with <32> P by random priming using a kit obtained from Boshringer Mannheim. Free nucleotides were removed by centrifugation through a Sephadex G-50 column. The specific activities of the probe was approximately 10<9 > cpm/ mu g.

Plaque hybridizations were carried out in 5 x SSC, 50% formamide, 2 x Denhardt's reagent, and 200 mu g/ml denatured salmon sperm DNA at 42o C. for 18-20 hours. Final washes were in 0.5 x SSC, 0.1% SDS at 65o C. Positive clones were identified after autoradiography.

EXAMPLE V

Construction of H Chain Genomic Library

To isolate the V region gene for the A2 H chain, a genomic library was constructed in the lambda gt10 vector system. High molecular weight DNA was digested to completion with restriction endonuclease EcoRI and fragments of approximately 7.5 kb were isolated after agarose gel electrophoresis. These fragments were ligated with lambda gt10 arms and packaged into phage particles in vitro using Gigapack Gold.

This library was screened at a density of 20,000 plaques per 150 mm plate using a J[H] probe. The J[H]probe was a 2 kb BamHI/EcoRI fragment containing both J3 and J4 segments. The probe was labeled as in Example III and had a similar specific radioactivity. Hybridization and wash conditions were identical to those used in Example III.

EXAMPLE VI

Cloning of the TNF-Specific V Gene Regions

Several positive clones were isolated from the H and L chain libraries after screening approximately 10<6 > plaques from each library using the J[H]and J[k]probes, respectively. Following plaque purification, bacteriophage DNA was isolated for each positive clone, digested with either EcoRI (H chain clones) or HindIII (L chain clones) and fractionated on 1% agarose gels. The DNA was transferred to nitrocellulose and the blots were hybridized with the J[H]or the J[K]probe.

Several H chain clones were obtained that contained 7.5 kb EcoRI DNA encoding fragments of MAbs to the J[H]probe. For the light chain libraries, several clones from each of the three size-selected libraries were isolated that contained HindIII fragments that hybridize to the J [k]probe. For the L chain, several independently derived HindIII fragments of 2.9 kb from the 2 kb library hybridized with a 1250 bp mRNA from A2, but not with SP2/0 mRNA (see Example VII). In addition, several HindIII fragments derived from the 4 kb library hybridized

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Method for preventing and treating hearing loss using a neuturin protein product

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CORE TERMS: cell, sequence, hair, neurturin, neuron, protein, amino acid, vector, polymer, variant, host, gene, residue, inner ear, nucleic acid, cochlea, cisplatin, spiral, promoter, ganglion, hearing loss, molecule, auditory, encoding, injection, medium, probè, ear, neurotrophic, membrane

ABST:

The present invention relates generally to methods for preventing and/or treating injury or degeneration of cochlear hair cells and spiral ganglion neurons by administering a neurturin neurotrophic factor protein product. The invention relates more specifically to methods for treating sensorineural hearing loss.

NO-OF-CLAIMS: 11

EXMPL-CLAIM: 1

NO-OF-FIGURES: 5

NO-DRWNG-PP: 5

PARCASE: This application claims the benefit of U.S. Provisional application No. 60/054,184 filed Jul. 30, 1997 which is hereby incorporated by reference.

SUM:

BACKGROUND OF THE INVENTION

The present invention relates generally to methods for preventing and/or treating injury or degeneration of inner ear sensory cells, such as hair cells and auditory neurons, by administering a neurotrophic factor protein product. The invention relates specifically to methods for preventing and/or treating hearing loss due to variety of causes.

Neurotrophic factors are natural proteins, found in the nervous system or in non-nerve tissues innervated by the nervous system, that function to promote the survival and maintain the phenotypic differentiation of certain nerve and/or glial cell populations (Varon et al., Ann. Rev. Neuroscience, 1:327, 1979; Thoenen et al., Science, 229:238, 1985). Because of this physiological role, certain neurotrophic factors have been found useful in treating the degeneration of certain nerve cells and the loss of differentiated function that results from nerve damage. Nerve damage is caused by conditions that compromise the survival and/or proper function of one or more types of nerve cells, including: (1) physical injury, which causes the degeneration of the axonal processes (which in turn causes nerve cell death) and/or nerve cell bodies near the site of injury, (2) temporary or permanent cessation of blood flow (ischemia) to parts of the nervous system, as in stroke, (3) intentional or accidental exposure to neurotoxins, such as the cancer and AIDS chemotherapeutic agents cisplatinum and dideoxycytidine, respectively, (4) chronic metabolic diseases, such as diabetes or renal dysfunction, or (5) neurodegenerative diseases such as Parkinson's disease, Alzheimer's disease, and Amyotrophic Lateral Sclerosis, which result from the degeneration of specific neuronal populations. In order for a particular neurotrophic factor to be potentially useful in treating nerve damage, the class or classes of damaged nerve cells must be responsive to the factor. It has been established that all neuron populations are not responsive to or equally affected by all neurotrophic factors.

The first neurotrophic factor to be identified was nerve growth factor (NGF). NGF is the first member of a defined family of trophic factors, called the neurotrophins, that currently includes brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), NT-4/5, and NT-6 (Thoenen, Trends. Neurosci., 14:165-170, 1991; Snider, Cell, 77:627-638, 1994; Bothwell, Ann. Rev. Neurosci., 18:223-253, 1995). These neurotrophins are known to act via the family of trk tyrosine kinase receptors, i.e., trkA, trkB, trkC, and the low affinity p75 receptor (Snider, Cell, 77:627-638, 1994; Bothwell, Ann. Rev. Neurosci., 18:223-253, 1995; Chao et al., TINS 18:321-326, 1995).

Glial cell line-derived neurotrophic factor (GDNF) is a protein identified and purified using assays based upon its efficacy in promoting the survival and stimulating the transmitter

phenotype of mesencephalic dopaminergic neurons in vitro (Lin et al., Science, 260:1130-1132, 1993). GDNF is a glycosylated disulfide-bonded homodimer that has some structural homology to the transforming growth factor-beta (TGF- beta) super family of proteins (Lin et al., Science, 260:1130-1132, 1993; Krieglstein et al., EMBO J., 14:736-742, 1995; Poulsen et al., Neuron, 13:1245-1252, 1994). GDNF mRNA has been detected in muscle and Schwann cells in the peripheral nervous system (Henderson et al., Science, 266:1062-1064, 1994; Trupp et al., J. Cell Biol., 130:137-148, 1995) and in type I astrocytes in the central nervous system (Schaar et al., Exp. Neurol., 124:368-371, 1993). In vivo, treatment with exogenous GDNF stimulates the dopaminergic phenotype of substantia nigra neurons and restores functional deficits induced by axotomy or dopaminergic neurotoxins in animal models of Parkinson's disease (Hudson et al., Brain Res. Bull., 36:425-432, 1995; Beck et al., Nature, 373:339-341, 1995; Tomac et al., Nature, 373:335-339, 1995; Hoffer et al., Neurosci. Lett., 182:107-111, 1994). Although originally thought to be relatively specific for dopaminergic neurons, at least in vitro, evidence is beginning to emerge indicating that GDNF may have a larger spectrum of neurotrophic targets besides mesencephalic dopaminergic and somatic motor neurons (Yan and Matheson, Nature 373:341-344, 1995; Oppenheim et al., Nature, 373:344-346, 1995; Matheson et al., Soc. Neurosci. Abstr, 21, 544, 1995; Trupp et al., J. Cell Biol., 130:137-148, 1995). In particular, GDNF was found to have neurotrophic efficacy on brainstem and spinal cord cholinergic motor neurons, both in vivo and in vitro (Oppenheim et al., Nature, 373:344-346, 1995; Zurn et al., Neuroreport, 6:113-118, 1994; Yan et al., Nature, 373: 341-344, 1995; Henderson et al., Science, 266:1062-1064, 1994), on retinal neurons, such as photoreceptors and retinal ganglion cells, and on sensory neurons from the dorsal root ganglion.

The neuroepithelial hair cells in the organ of Corti of the inner ear, transduce sound into neural activity, which is transmitted along the cochlear division of the eighth cranial nerve. This nerve consists of fibers from three types of neurons (Spoendlin, H. H. In: Friedmann, I. Ballantyne, J., eds. Ultrastructural Atlas of the Inner Ear; London, Butterworth, pp. 133-164, 1984): 1) afferent neurons, which lie in the spiral ganglion and connect the cochlea to the brainstem. 2) efferent olivocochlear neurons, which originate in the superior olivary complex and 3) autonomic adrenergic neurons, which originate in the cervical sympathetic trunk and innervate the cochlea. In the human, there are approximately 30,000 afferent cochlear neurons, with myelinated axons, each consisting of about 50 lamellae, and 4-6 mu m in diameter. This histologic structure forms the basis of uniform conduction velocity, which is an important functional feature. Throughout the length of the auditory nerve, there is a trophic arrangement of afferent fibers, with 'basal' fibers wrapped over the centrally placed 'apical' fibers in a twisted rope-like fashion. Spoendlin (Spoendlin, H. H. In: Naunton, R. F., Fernadex, C. eds. Evoked Electrical Activity in the Auditory Nervous System. London, Academic Press, pp. 21-39, 1978) identified two types of afferent neurons in the spiral ganglion on the basis of morphologic differences: type I cells (95%) are bipolar and have myelinated cell bodies and axons that project to the inner hair cells. Type II cells (5%) are monopolar with unmyelinated axons and project to the outer hair cells of the organ of Corti. Each inner hair cell is innervated by about 20 fibers, each of which synapses on only one cell. In contrast, each outer hair cell is innervated by approximately six fibers, and each fiber branches to supply approximately 10 cells. Within the cochlea, the fibers divide into: 1) an inner spiral group, which arises primarily ipsilaterally and synapses with the afferent neurons to the inner hair cells, and 2) a more numerous outer radial group, which arises mainly contralaterally and synapses directly with outer hair cells. There is a minimal threshold at one frequency, the characteristic or best frequency, but the threshold rises sharply for frequencies above and below this level (Pickles, J. O. In: Introduction to the Physiology of Hearing. London, Academic Press, pp. 71-106, 1982). Single auditory nerve fibers therefore appear to behave as band-pass filters. The basilar membrane vibrates preferentially to different frequencies, at different distances along its length, and the frequency selectivity of each cochlear nerve fiber is similar to that of the inner hair cell to which the fiber is connected. Thus, each cochlear nerve fiber exhibits a turning curve covering a different range of frequencies from its neighboring fiber (Evans, E. F. In: Beagley H. A. ed. Auditory

investigation: The Scientific and Technological basis. New York, Oxford University Press, 1979). By this mechanism, complex sounds are broken down into component frequencies (frequency resolution) by the filters of the inner ear.

Hearing loss of a degree sufficient to interfere with social and job-related communications is among the most common chronic neural impairments in the U.S. population. On the basis of health-interview data (Vital and health statistics. Series 10. No. 176. Washington, D.C. (DHHS publication no. (PHS) 90-1504), it is estimated that approximately 4 percent of people under 45 years of age and about 29 percent of those 65 years or over have a handicapping loss of hearing. It has been estimated that more than 28 million Americans have hearing impairment and that as many as 2 million of this group are profoundly deaf (A report of the task force on the National Strategic plan. Bethesda, Md.: National Institute of Health, 1989). The prevalence of hearing loss increases dramatically with age. Approximately 1 per 1000 infants has a hearing loss sufficiently severe to prevent the unaided development of spoken language (Gentile, A. et al., Characteristics of persons with impaired hearing: United States, 1962-1963. Series 10. No. 35. Washington, D.C.: Government printing office, 1967 (DHHS publication no. (PHS) 1000) (Human communication and its disorders: an overview. Bethesda, Md.: National Institutes of health, 1970). More than 360 per 1000 persons over the age of 75 have a handicapping hearing loss (Vital and health statistics. Series 10. No. 176. Washington, D.C. (DHHS publication no. (PHS) 90-1504).

It has been estimated that the cost of lost productivity, special education, and medical treatment may exceed $ 30 billion per year for disorders of hearing, speech and language (1990 annual report of the National Deafness and other Communication Disorders Advisory Board. Washington, D.C.: Government Printing Office, 1991. (DHHS publication no. (NIH) 91-3189). The major common causes of profound deafness in childhood are genetic disorders and meningitis, constituting approximately 13 percent and 9 percent of the total, respectively (Hotchkiss, D. Demographic aspects of hearing impairment: questions and answers. 2nd ed. Washington, D.C.: Gallaudet University Press, 1989). In approximately 50 percent of the cases of childhood deafness, the cause is unknown, but is likely due to genetic causes or predisposition (Nance W E, Sweeney A. Otolaryngol. Clin. North Am 1975; 8: 19-48).

Impairment anywhere along the auditory pathway, from the external auditory canal to the central nervous system, may result in hearing loss. The auditory apparatus can be subdivided into the external and middle ear, inner ear and auditory nerve and central auditory pathways. Auditory information in humans is transduced from a mechanical signal to a neurally conducted electrical impulse by the action of approximately 15,000 neuroepithelial cells (hair cells) and 30,000 first-order neurons (spiral ganglion cells) in the inner ear. All central fibers of spiral ganglion neurons form synapses in the cochlear nucleus of the pontine brainstem. The number of neurons involved in hearing increases dramatically from the cochlea to the auditory brain stem and the auditory cortex. All auditory information is transduced by only 15,000 hair cells, of which the so-called inner hair cells, numbering 3500, are critically important, since they form synapses with approximately 90 percent of the 30,000 primary auditory neurons. Thus, damage to a relatively few cells in the auditory periphery can lead to substantial hearing loss. Hence, most causes of sensorineural loss can be ascribed to lesions in the inner ear (Nadol, J. B., New England Journal of Medicine, 1993, 329: 1092-1102).

Hearing loss can be on the level of conductivity, sensorineural and central level. Conductive hearing loss is caused by lesions involving the external or middle ear, resulting in the destruction of the normal pathway of airborne sound amplified by the tympanic membrane and the ossicles to the inner ear fluids. Sensorineural hearing loss is caused by lesions of the cochlea or the auditory division of the eight cranial nerve. Central hearing loss is due to lesions of the central auditory pathways. These consist of the cochlear and dorsal olivary nucleus complex, inferior colliculi, medial geniculate bodies, auditory cortex in the temporal lobes and interconnecting afferent and efferent fiber tracts (Adams R. D. and Maurice, V. Eds.

in: Principles of Neurology. 1989. McGraw-Hill Information services Company. PP 226-246).

As mentioned previously, at least 50 percent of cases of profound deafness in childhood have genetic causes (Brown, K. S., Med. Clin. North AM. 1969; 53:741-72). If one takes into consideration the probability that genetic predisposition is a major causative factor in presbycusis- or age-related hearing loss which affects one third of the population over 75 years of age (Nadol, J. B. In: Beasley D S, Davis G A, eds. Aging: Communication Processes and Disorders. New York: Grune & Stratton, 1981:63-85), genetic and hereditary factors are probably the single most common cause of hearing loss. Genetic anomalies are much more commonly expressed as sensorineural hearing loss than as conductive hearing loss. Genetically determined sensorineural hearing loss is clearly a major, if not the main cause of sensorineural loss, particularly in children (Nance W E, Sweeney A. Otolaryngol. Clin. North Am 1975; 8: 19-48). Among the most common syndromal forms of sensorineural loss are Waardenburg's syndrome, Alport's syndrome and Usher's syndrome.

A variety of commonly used drugs have ototoxic properties. The best known are the aminoglycoside antibiotics (Lerner, S. A. et al. eds. Aminoglycoside ototoxicity. Boston: Little, Brown, 1981; Smith, C. R. et al. N Engl. J. Med. 1980; 302: 1106-9), loop diuretics (Bosher, S. K., Acta Otolaryngol. (Stockholm) 1980; 90: 4-54), salicylates (Myers, E. N. et al., N Engl. J. Med. 1965; 273:587-90) and antineoplastic agents such as cisplatin (Strauss, (Strauss, M. et al., Laryngoscope 1983; 143:1263 -5). Ototoxicity has also been described during oral or parenteral administration of erythromycin (Kroboth, P. D. et al., Arch. Intern Med. 1983; 1: 169-79; Achweitzer, V. G., Olson, N. Arch. Otolaryngol. 1984; 110:258-60).

Most ototoxic substances cause hearing loss by damaging the cochlea, particularly the auditory hair cells and the stria vascularis, a specialized epithelial organ within the inner ear, that is responsible for the homeostasis of fluids and electrolytes (Nadol, J. B. New England J. Med. 1993, 329: 1092-1102). Secondary neural degeneration may occur many years after an ototoxic event affecting the hair cells. There is evidence that some ototoxic substances may be selectively concentrated within the inner ear, resulting in progressive sensorineural loss despite the discontinuation of systemic administration (Federspil, P. et al., J. Infect. Dis. 1976; 134 Suppl: S200-S205)

Trauma due to acoustic overstimulation is another leading cause of deafness. There is individual susceptibility to trauma from noise. Clinically important sensorineural hearing loss may occur in some people exposed to high-intensity noise, even below levels approved by the Occupational Safety and Health Agency (Osguthorpe, J. D. ed. Washington D.C.: American Academy of Otolaryngology-Head and Neck Surgery Foundation, 1988).

Demyelinating processes, such as multiple sclerosis, may cause sensorineural hearing loss (Noffsinger, D et al., Acta Otolaryngol Suppl (Stockh) 1972; 303:1-63). More recently, a form of immune-mediated sensorineural hearing loss has been recognized (McCabe, B. F. Ann Otol Rhinol Laryngol 1979; 88:585-9). The hearing loss is usually bilateral, is rapidly progressive (measured in weeks and months), and may or may not be associated with vestibular symptoms.

A variety of tumors, both primary and metastatic, can produce either a conductive hearing loss, or a sensorineural hearing loss, by invading the inner ear or auditory nerve (Houck, J. R. et al., Otolaryngol Head Neck Surg 1992; 106:92-7). A variety of degenerative disorders of unknown cause can produce sensorineural hearing loss. Meniere's syndrome (Nadol, J. B. ed. Meniere's disease: pathogenesis, pathophysiology, diagnosis, and treatment. Amsterdam: Kugler & Ghedini 1989), characterized by fluctuating sensorineural hearing loss, episodic vertigo, and tinnitus, appears to be caused by a disorder of fluid homeostasis within the inner ear, although the pathogenesis remains unknown. Sudden idiopathic sensorineural hearing loss (Wilson, W. R. et al., Arch Otolaryngol 1980; 106:772-6), causing moderate-to-severe sensorineural deafness, may be due to various causes, including inner ear ischemia

and viral labyrinthitis.

Presbycusis, the hearing loss associated with aging, affects more than one third of persons over the age of 75 years. The most common histopathological correlate of presbycusis is the loss of neuroepithelial (hair) cells, neurons, and the stria vascularis of the peripheral auditory system (Schuknecht H. F. Pathology of the Ear. Cambridge, Mass: Harvard University Press, 1974:415-420). Presbycusis is best understood as resulting from the cumulative effects of several noxious influences during life, including noise trauma, ototoxicity and genetically influenced degeneration.

Certain neurotrophic factors have been shown to regulate neuronal differentiation and survival during development (Korsching S. J. Neurosci. 13:2739-2748,1993) and to protect neurons from injury and toxins in adult (Hefti, Neurosci. 6:2155-2162, 1986; Apfel et al., Ann Neurol 29:87-89, 1991; Hyman et al., Nature 350:230-233, 1991; Knusel et al., J. Neurosci. 12:4391-4402, 1992; Yan et al., Nature, 360:753-755, 1992; Koliatsos et al., Neuron, 10:359-367, 1993). In situ hybridization studies indicate that mRNAs for the neurotrophin receptors TrkB and TrkC are expressed by developing cochleovestibular ganglia (Ylikoski et al., Hear. Res. 65:69-78 1993; Schecterson et al., Hearing Res. 73: 92-100 1994) and that mRNAs for BDNF and NT-3 are found in the inner ear, including the organ of Corti (Pirvola et al., Proc. Natl. Acad. Sci. USA 89: 9915-9919, 1992; Schecterson et al., Hearing Res. 73: 92-100 1994; Wheeler et al., Hearing Res. 73: 46-56, 1994). The physiological role of BDNF and NT-3 in the development of the vestibular and auditory systems was investigated in mice that carry a deleted BDNF and/or NT-3 gene (Ernfors et al., Neuron 14: 1153-1164 1995). In the cochlea, BDNF mutants lost type-2 spiral neurons, causing an absence of outer hair cell innervation. NT-3 mutants showed a paucity of afferents and lost 87 percent of spiral neurons, presumably corresponding to type-1 neurons, which innervate inner hair cells. Double mutants had an additive loss, lacking all vestibular and spiral neurons. The requirement of TrkB and TrkC receptors for the survival of specific neuronal populations and the maintenance of target innervation in the peripheral sensory system of the inner ear was demonstrated by studying mice carrying a germline mutation in the tyrosine kinase catalytic domain of these genes (Schimmang et al., Development, 121: 3381-3391 1995). Gao et al., (J. Neurosci. 15: 5079-5087, 1995) showed survival-promoting potency of NT-4/5, BDNF and NT-3 for rat postnatal spiral ganglion neurons in dissociated cultures and that NT-4/5 protected these neurons from neurotoxic effects of the anti-cancer drug, cisplatin. Also, BDNF and NT-3 have been shown to support the survival of adult rat auditory neurons in dissociated cultures (Lefebvre et al., Neuroreport 5: 865-868, 1994).

There have been no previous reports of the use of neurturin in the treatment of hearing loss. Since hearing impairment is a serious affliction, the identification of any agent and treatment method that can protect the auditory neurons and hair calls from damage would be of great benefit.

SUMMARY OF THE INVENTION

The present invention provides methods for treating sensorineural hearing loss comprising administering to a subject having a lesion in the inner ear a therapeutically effective amount of a neurturin neurotrophic factor protein product. For example, the hearing loss may be associated with injury or degeneration of neuroepithelial hair cells (cochlear hair cells) or spiral ganglion neurons in the inner ear.

The present invention is based on the discoveries that hair cells respond to neurturin by resisting the toxic effects of ototoxins, such as cisplatin and neomycin, and that auditory neurons also respond to neurturin by resisting the toxic effects of variety of ototoxins, such as for example cisplatin, neomycin, and sodium salicylate. Thus, a therapeutically effective amount neurturin protein product may be administered to promote the protection, survival or

regeneration of hair cells and spiral ganglion neurons.

It has also been discovered that lesions or disturbances to the vestibular apparatus may also be treated by administering to a subject having such a lesion or disturbance a therapeutically effective amount of a neurturin protein product. Such lesions may result in dizziness, vertigo or loss of balance.

It is contemplated that such neurturin protein products would preferably include a neurturin protein such as that depicted by the amino acid sequence set forth in the Figures, as well as variants and derivatives thereof. It is also contemplated that such neurturin protein products would include [Met< - 1>]neurturin.

According to the invention, the neurturin protein product may be administered parenterally at a dose ranging from about 1 mu g/kg/day to about 100 mg/kg/day, typically at a dose of about 0.1 mg/kg/day to about 25 mg/kg/day, and usually at a dose of about 5 mg/kg/day to about 20 mg/kg/day. It is also contemplated that, depending on the individual patient's needs and route of administration, the neurturin protein product may be given at a lower frequency such as weekly or several times per week, rather than daily. It is further contemplated that neurturin protein product may be administered directly into the middle ear or the inner ear. One skilled in the art will appreciate that with such administration of a smaller amount of neurturin protein product may be used, for example, a direct middle ear or inner-ear administration dose in the range of about 1 mu g/ear to about 1 mg/ear in a single injection or in multiple injections. Alternatively, if administered topically or orally, a comparatively larger dose may be used.

It is further contemplated that neurturin protein product be administered in combination or conjunction with an effective amount of a second therapeutic agents, such as GDNF, BDNF and NT-3. The invention also provides for the use of neurturin protein product in the manufacture of a medicament or pharmaceutical composition for the treatment of injury or degeneration of hair cells and auditory neurons for the variety of causes of sensorineural hearing loss. Such pharmaceutical compositions include topical, oral or middle and inner ear neurturin protein product formulations or in combination with cochlear implants.

It will also be appreciated by those skilled in the art that the administration process can be accomplished via cell therapy and gene therapy means, as further described below. For example, in a gene therapy means cells have been modified to produce and secrete the neurturin protein product. The cells may be modified ex vivo or in vivo. Numerous additional aspects and advantages of the invention will become apparent to those skilled in the art upon consideration of the following detailed description of the invention which describes presently preferred embodiments thereof.

DRWDESC:
BRIEF DESCRIPTION OF THE FIGS.

Numerous features and advantages of the present invention will become apparent upon review of the figures, wherein:

FIG. 1 depicts an amino acid sequence (SEQ ID NO: 1) of human neurturin neurotrophic factor.

FIG. 2 depicts an amino acid sequence (SEQ ID NO: 2) of mouse neurturin neurotrophic factor.

FIG. 3 depicts a nucleic acid sequence (SEQ ID NO: 3) encoding a neurturin neurotrophic factor analog.

FIG. 4 depicts an amino acid sequence (SEQ ID NOs: 3 or 4) of a human neurturin neurotrophic factor analog.

FIG. 5 depicts an amino acid sequence (SEQ ID NO: 5) of pre-pro human neurturin neurotrophic factor.

DETDESC:

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a method for preventing and/or treating sensorineural hearing loss by administering a therapeutically effective amount of a neurturin neurotrophic factor protein product. According to one aspect of the invention, methods are provided for treating damaged hair cells and auditory neurons by administering a therapeutically effective amount of neurturin protein product by means of a pharmaceutical composition, the implantation of neurturin-expressing cells, or neurturin gene therapy. The invention may be practiced using a biologically active neurturin protein product, including the proteins represented by the amino acid sequences set forth in FIGS. 1, 2, 4 and 5 (SEQ ID NOs: 1, 2, 3, 4 and 5), including variants and derivatives thereof. In addition to oral, parenteral or topical delivery of the neurturin protein product, administration via cell therapy and gene therapy procedures is contemplated.

The present invention is based on the initial discoveries that neurturin protects hair cells from ototoxin-induced cell death in explant cultures of rat's cochlea and dissociated spiral ganglion neurons from adult rat in culture. It is contemplated that administration of a neurturin protein product will protect hair cells and spiral ganglion neurons from traumatic damage (such as noise trauma and acute or chronic treatments of cisplatin and aminoglycoside antibiotics) or from damage resulting from a lack of neurotrophic factors caused by interruption of transport of the factors from the axon to the cell body. Such treatment is expected to allow hair cells and/or auditory neurons to tolerate intermittent insults from trauma or ototoxins and to slow down the progressive degeneration of the auditory neurons and hair cells that is responsible for hearing loss in pathological conditions such as presbycusis (age-related hearing loss), inherited sensorineural degeneration, and post-idiopathic hearing losses and to preserve the functional integrity of the inner ear. It will also support the auditory neurons for a better and longer performance of cochlear implants.

According to the invention, the neurturin protein product may be administered into the middle ear at a dose ranging from about 1 mu g/kg/day to about 100 mg/kg/day, typically at a dose of about 0.1 mg/kg/day to about 25 mg/kg/day, and usually at a dose of about 5 mg/kg/day to about 20 mg/kg/day. A neurturin protein product may be administered directly into the inner ear in cases where invasion of the inner ear is already in place such as in the procedure of cochlear implant or surgeries of the inner ear. In such cases, a smaller amount of neurturin protein product will be administered, for example, from about 1 mu g/ear to about 1 mg/ear in a single injection or in multiple injections. In situations where the chronic administration of the protein product is needed, a delivery device such as an Alzet mini-pump may be attached to a cannula the tip of which will be introduced into the middle or inner ear for a continuous release of the protein product. Alternatively, a neurturin protein product may be delivered in the form of ear-drops which will penetrate the tympanic membrane of the Bulla. It is further contemplated that a neurturin protein product may be administered together with an effective amount of a second therapeutic agent for the treatment of auditory neuron degeneration, for example GDNF, BDNF and NT-3 as well as other neurotrophic factors or drugs used in the treatment of various inner ear pathologies. A variety of pharmaceutical formulations and different delivery techniques are described in further detail below.

As used herein, the term "neurturin protein product" includes purified natural, synthetic or

recombinant neurturin neurotrophic factor, biologically active neurturin variants (including insertion, substitution and deletion variants), and chemically modified derivatives thereof. Also included are neurturin proteins that are substantially homologous to the human neurturin protein having the amino acid sequence set forth in FIGS. 1 and 4 (SEQ ID NOs: 1, 3 and 4). In addition, chemically modified derivatives of these various proteins are included in the present invention. Neurturin protein products also may exist as homodimers or heterodimers in their biologically active form.

The term "biologically active" as used herein means that the neurturin protein product demonstrates similar neurotrophic properties, but not necessarily all of the same properties, and not necessarily to the same degree, as the neurturin having the amino acid sequence set forth in the Figures, but having at least the activity of promoting the protection, survival or regeneration of hair cells and spiral ganglion neurons. The selection of the particular neurotrophic properties of interest depends upon the use for which the neurturin protein product is being administered.

The term "substantially homologous" as used herein means having a degree of homology to the neurturin protein having the amino acid sequence set forth in FIGS. 1, 2, 4 and 5 (SEQ ID NO: 1, 2, 3, 4 and 5) that is preferably in excess of 70%, most preferably in excess of 80%, and even more preferably in excess of 90% or 95%. For example, the degree of homology between the mouse and the human protein is about 91%, and it is contemplated that preferred mammalian neurturin proteins will have a similarly high degree of homology. The percentage of homology or percent identity is calculated as the percentage of amino acid residues found in the smaller of the two sequences which align with identical amino acid residues in the sequence being compared, when four gaps in a length of 100 amino acids may be introduced to assist in that alignment (as set forth by Dayhoff, in Atlas of Protein Sequence and Structure, Vol. 5, p. 124, National Biochemical Research Foundation, Washington, D.C. (1972), the disclosure of which is hereby incorporated by reference). Also included as substantially homologous is any neurturin protein product which may be isolated by virtue of cross-reactivity with antibodies to the neurturin of FIGS. 1 or 2 (SEQ ID NO: 1 or 2) or whose genes may be isolated through hybridization with the gene or with segments of the gene encoding the neurturin of FIG. 1 or 2 (SEQ ID NO: 1 or 2).

The neurturin protein products according to this invention may be isolated or generated by a variety of means. Exemplary methods for producing neurturin protein products useful in the present invention are substantially similar to the methods of producing GDNF as described in U.S. application Ser. No. 08/182,183 filed May 23, 1994 and its parent applications; PCT Application No. PCT/US92/07888 filed Sep. 17, 1992, published as WO 93/06116 (Lin et al., Syntex-Synergen Neuroscience Joint Venture); European Patent Application No. 92921022.7, published as EP 610 254; and U.S. application Ser. No. 08/535,681 filed Sep. 28, 1995 ("Truncated Glial Cell-Line Derived Neurotrophic Factor"), the disclosures of which are hereby incorporated by reference.

Neurturin protein products may be chemically or recombinantly synthesized by means known to those skilled in the art, see for example Kotzbauer et al., Nature 384:467-470, 1996. Neurturin protein products are preferably produced via recombinant techniques because such methods are capable of achieving comparatively higher amounts of protein at a greater purity. Recombinant neurturin protein product forms include glycosylated and non-glycosylated forms of the protein, and include but are not limited to protein product expressed in bacterial, mammalian or insect cell systems.

In general, recombinant techniques involve isolating the genes responsible for coding neurturin, cloning the gene in suitable vectors and/or cell types, modifying the gene if necessary to encode a desired variant, and expressing the gene in order to produce the neurturin protein product. Alternatively, a nucleotide sequence encoding the desired neurturin protein product may be chemically synthesized. It is contemplated that a neurturin

protein product may be expressed using nucleotide sequences which vary in codon usage due to the degeneracies of the genetic code or allelic variations or alterations made to facilitate production of the protein product by the select cell. Kotzbauer et al., Nature 384:467-470, describes the identification of a mouse cDNA and amino acid sequence and a human cDNA and amino acid sequence for neurturin protein. WO93/06116 describes a variety of vectors, host cells, and culture growth conditions for the expression of GDNF protein product which may also be used to express the neurturin protein product. Additional vectors suitable for the expression of neurturin protein product in E. coli are disclosed in published European Patent Application No. EP 0 423 980 ("Stem Cell Factor") published Apr. 24, 1991, the disclosure of which is hereby incorporated by reference.

The molecular weight of purified neurturin indicates that the protein is a disulfide-bonded dimer in its biologically active form. The material isolated after expression in a bacterial system is essentially biologically inactive, and exists as a monomer. Refolding is necessary to produce the biologically active disulfide-bonded dimer. Processes suitable for the refolding and naturation of the neurturin expressed in bacterial systems are substantially similar to those described in WO93/06116. Standard in vitro assays for the determination of neurturin activity are also substantially similar to those determining GDNF activity as described in WO93/06116 and in co-owned, co-pending U.S. application Ser. No. 08/535,681 filed Sep. 28, 1995, and are hereby incorporated by reference.

A. Neurturin Variants

The term "neurturin variants" as used herein includes polypeptides in which one or more amino acids have been deleted from ("deletion variants"), inserted into ("addition variants"), or substituted for ("substitution variants"), residues within the amino acid sequence of neurturin of FIGS. 1, 2, 4 and 5. Such variants are prepared by introducing appropriate nucleotide changes into the DNA encoding the polypeptide or by in vitro chemical synthesis of the desired polypeptide. It will be appreciated by those skilled in the art that many combinations of deletions, insertions, and substitutions can be made provided that the final molecule possesses neurturin biological activity. An exemplary substitution variant is depicted in FIG. 4.

Mutagenesis techniques for the replacement, insertion or deletion of one or more selected amino acid residues are well known to one skilled in the art (e.g., U.S. Pat. No. 4,518,584, the disclosure of which is hereby incorporated by reference.) There are two principal variables in the construction of variants: the location of the mutation site and the nature of the mutation. In designing neurturin variants, the selection of the mutation site and nature of the mutation may depend on the neurturin characteristic(s) to be modified. The sites for mutation can be modified individually or in series, e.g., by (1) substituting first with conservative amino acid choices and then with more radical selections depending upon the results achieved, (2) deleting the target amino acid residue, or (3) inserting amino acid residues adjacent to the located site. Conservative changes in from 1 to 20 amino acids are preferred. Once the amino acid sequence of the desired neurturin protein product is determined, the nucleic acid sequence to be used in the expression of the protein is readily determined. N-terminal and C-terminal deletion variants may also be generated by proteolytic enzymes.

For neurturin deletion variants, deletions generally range from about 1 to 30 residues, more usually from about 1 to 10 residues, and typically from about 1 to 5 contiguous residues. N-terminal, C-terminal and internal intrasequence deletions are contemplated. Deletions may be introduced into regions of low homology with other TGF- beta super family members to modify the activity of neurturin. Deletions in areas of substantial homology with other TGF-beta super family sequences will be more likely to modify the neurturin biological activity more significantly. The number of consecutive deletions will be selected so as to preserve the tertiary structure of the neurturin protein product in the affected domain, e.g., cysteine

crosslinking. Non-limiting examples of deletion variants include truncated neurturin protein products lacking from one to seven N-terminal amino acids, or variants lacking the C-terminal residue, or combinations thereof.

In a basic embodiment, the truncated neurturin proteins may be represented by the following amino acid sequence wherein the amino acid residue numbering scheme of FIG. 1 is used to facilitate comparison to the human neurturin protein:

X-[Cys<8> -Cys<101>]-Y

wherein

[Cys<8> -Cys<101>] represents the amino acid sequence of Cys<8 > through Cys<101 > as depicted in FIG. 1 (SEQ ID NO: 1);

Y represents the zero or one or more carboxy-terminus amino acid residues, for example, Val<102> ; and

X represents zero, a methionine residue or one or more amino-terminus amino acid residues, for example:

```
                            P
                           RP
                          ARP
                         GARP
                        LGARP
                       RLGARP
                      ARLGARP
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As used herein, the term "truncated neurturin protein product" includes biologically active synthetic or recombinant truncated neurturin proteins, truncated neurturin proteins produced from mature neurturin, biologically active truncated neurturin variants (including insertion, substitution and deletion variants), and chemically modified derivatives thereof. Also included are truncated neurturin proteins that are substantially homologous to the human neurturin protein having the amino acid sequence set forth in FIG. 1 (SEQ ID NO: 1).

For neurturin addition variants, amino acid sequence additions typically include N- and/or C-terminal fusions ranging in length from one residue to polypeptides containing a hundred or more residues, as well as internal intrasequence additions of single or multiple amino acid residues. Internal additions may range generally from about 1 to 10 residues, more typically from about 1 to 5 residues, and usually from about 1 to 3 amino acid residues. Examples of N-terminal addition variants include neurturin with an N-terminal methionyl residue (for example, an artifact of the direct expression of neurturin in bacterial recombinant cell culture), which is designated [Met< - 1>]neurturin, and fusion of a heterologous N-terminal signal sequence to the N-terminus of neurturin to facilitate the secretion of mature neurturin from recombinant host cells. Such signal sequences generally will be obtained from, and thus be homologous to, the intended host cell species. Additions may also include amino acid sequences derived from the sequence of other neurotrophic factors, for example, from 1 to 35 N-terminal amino acid residues of the human or rat GDNF proteins. A preferred neurturin protein product for use according to the present invention is the recombinant human [Met< - 1>]neurturin.

Neurturin substitution variants have at least one amino acid residue of the human or mouse neurturin amino acid sequence removed and a different residue inserted in its place. Such substitution variants include allelic variants, which are characterized by naturally-occurring nucleotide sequence changes in the species population that may or may not result in an amino acid change. An example of a substitution variant is depicted in FIG. 4 (SEQ ID NOs: 3 or 4).

Specific mutations of the neurturin amino acid sequence may involve modifications to a glycosylation site (e.g., serine, threonine, or asparagine). The absence of glycosylation or only partial glycosylation results from amino acid substitution or deletion at any asparagine-linked glycosylation recognition site or at any site of the molecule that is modified by addition of an O-linked carbohydrate. An asparagine-linked glycosylation recognition site comprises a tripeptide sequence which is specifically recognized by appropriate cellular glycosylation enzymes. These tripeptide sequences are either Asn-Xaa-Thr or Asn-Xaa-Ser, where Xaa can be any amino acid other than Pro. A variety of amino acid substitutions or deletions at one or both of the first or third amino acid positions of a glycosylation recognition site (and/or amino acid deletion at the second position) result in non-glycosylation at the modified tripeptide sequence. Thus, the expression of appropriate altered nucleotide sequences produces variants which are not glycosylated at that site. Alternatively, the neurturin amino acid sequence may be modified to add glycosylation sites.

One method for identifying neurturin amino acid residues or regions for mutagenesis is called "alanine scanning mutagenesis" as described by Cunningham and Wells (Science, 244:1081-1085, 1989). In this method, an amino acid residue or group of target residues are identified (e.g., charged residues such as Arg, Asp, His, Lys, and Glu) and replaced by a neutral or negatively charged amino acid (most preferably alanine or polyalanine) to affect the interaction of the amino acids with the surrounding aqueous environment in or outside the cell. Those domains demonstrating functional sensitivity to the substitutions then are refined by introducing additional or alternate residues at the sites of substitution. Thus, the target site for introducing an amino acid sequence variation is determined, alanine scanning or random mutagenesis is conducted on the corresponding target codon or region of the DNA sequence, and the expressed neurturin variants are screened for the optimal combination of desired activity and degree of activity.

The sites of greatest interest for substitutional mutagenesis include sites where the amino acids found in neurturin proteins from various species are substantially different in terms of side-chain bulk, charge, and/or hydrophobicity. Other sites of interest are those in which particular residues of neurturin-like proteins, obtained from various species, are identical. Such positions are generally important for the biological activity of a protein. Initially, these sites are substituted in a relatively conservative manner. Such conservative substitutions are shown in Table 1 under the heading of preferred substitutions. If such substitutions result in a change in biological activity, then more substantial changes (exemplary substitutions) are introduced, and/or other additions or deletions may be made, and the resulting products screened for activity.

TABLE 1

Amino Acid Substitutions

* Original Residue	Preferred Substitutions	Exemplary Substitutions
Ala (A)	Val	Val; Leu; Ile
Arg (R)	Lys	Lys; Gln; Asn
Asn (N)	Gln	Gln; His; Lys; Arg
Asp (D)	Glu	Glu
Cys (C)	Ser	Ser

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Gln (Q)              Asn          Asn
Glu (E)              Asp          Asp
Gly (G)              Pro          Pro
His (H)              Arg          Asn; Gln; Lys; Arg
Ile (I)              Leu          Leu; Val; Met; Ala; Phe;
                       *           norleucine
Leu (L)              Ile          norleucine; Ile; Val; Met;
                       *           Ala; Phe
Lys (K)              Arg          Arg; Gln; Asn
Met (M)              Leu          Leu; Phe; Ile
Phe (F)              Leu          Leu; Val; Ile; Ala
Pro (P)              Gly          Gly
Ser (S)              Thr          Thr
Thr (T)              Ser          Ser
Trp (W)              Tyr          Tyr
Tyr (Y)              Phe          Trp; Phe; Thr; Ser
Val (V)              Leu          Ile; Leu; Met; Phe; Ala;
                       *           norleucine
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Conservative modifications to the amino acid sequence (and the corresponding modifications to the encoding nucleic acid sequences) are expected to produce neurturin protein products having functional and chemical characteristics similar to those of natural neurturin. In contrast, substantial modifications in the functional and/or chemical characteristics of neurturin protein products may be accomplished by selecting substitutions that differ significantly in their effect on maintaining (a) the structure of the polypeptide backbone in the area of the substitution, for example, as a sheet or helical conformation, (b) the charge or hydrophobicity of the molecule at the target site, or (c) the bulk of the side chain. Naturally occurring residues are divided into groups based on common side chain properties:

1) hydrophobic: norleucine, Met, Ala, Val, Leu, Ile;

2) neutral hydrophilic: Cys, Ser, Thr;

3) acidic: Asp, Glu;

4) basic: Asn, Gln, His, Lys, Arg;

5) residues that influence chain orientation: Gly, Pro; and

6) aromatic: Trp, Tyr, Phe.

Non-conservative substitutions may involve the exchange of a member of one of these classes for another. Such substituted residues may be introduced into regions of the neurturin protein that are homologous with other TGF- beta super family proteins including GDNF, or into the non-homologous regions of the molecule.

B. Neurturin Derivatives

Chemically modified derivatives of neurturin protein products also may be prepared by one of

skill in the art given the disclosures herein. The chemical moieties most suitable for derivatization include water soluble polymers. A water soluble polymer is desirable because the protein to which it is attached does not precipitate in an aqueous environment, such as a physiological environment. Preferably, the polymer will be pharmaceutically acceptable for the preparation of a therapeutic product or composition. One skilled in the art will be able to select the desired polymer based on such considerations as whether the polymer/protein conjugate will be used therapeutically, and if so, the desired dosage, circulation time, resistance to proteolysis, and other considerations.

Suitable water soluble polymers include, but are not limited to, polyethylene glycol (PEG), copolymers of ethylene glycol/propylene glycol, carboxymethylcellulose, dextran, polyvinyl alcohol, polyvinyl pyrrolidone, poly-1,3-dioxolane, poly-1,3,6-trioxane, ethylene/maleic anhydride copolymer, polyaminoacids (either homopolymers or random copolymers), and dextran or poly(n-vinyl pyrrolidone)polyethylene glycol, propropylene glycol homopolymers, polypropylene oxide/ethylene oxide co-polymers, polyoxyethylated polyols (e.g., glycerol), polyvinyl alcohol, and mixtures thereof. Polyethylene glycol propionaldehyde may have advantages in manufacturing due to its stability in water.

The polymer may be of any molecular weight, and may be branched or unbranched. For polyethylene glycol, the preferred molecular weight ranges from about 2 kDa to about 100 kDa for ease in handling and manufacturing (the term "about" indicating that in preparations of polyethylene glycol, some molecules will weigh more, some less, than the stated molecular weight). Other sizes may be used, depending on the desired therapeutic profile (e.g., the duration of sustained release desired, the effects, if any on biological activity, the ease in handling, the degree or lack of antigenicity and other known effects of polyethylene glycol on a therapeutic protein or variant).

The number of polymer molecules so attached may vary, and one skilled in the art will be able to ascertain the effect on function. One may mono-derivatize, or may provide for a di-, tri-, tetra- or some combination of derivatization, with the same or different chemical moieties (e.g., polymers, such as different weights of polyethylene glycols). The proportion of polymer molecules to protein (or peptide) molecules will vary, as will their concentrations in the reaction mixture. In general, the optimum ratio (in terms of efficiency of reaction in that there is no excess unreacted protein or polymer) will be determined by factors such as the desired degree of derivatization (e.g., mono-, di-, tri-, etc.), the molecular weight of the polymer selected, whether the polymer is branched or unbranched, and the reaction conditions.

The polyethylene glycol molecules (or other chemical moieties) should be attached to the protein with consideration of effects on functional or antigenic domains of the protein. There are a number of attachment methods available to those skilled in the art. See for example, EP 0 401 384, the disclosure of which is hereby incorporated by reference (coupling PEG to G-CSF), see also Malik et al., Exp. Hematol., 20:1028-1035, 1992 (reporting pegylation of GM-CSF using tresyl chloride). For example, polyethylene glycol may be covalently bound through amino acid residues via a reactive group, such as, a free amino or carboxyl group. Reactive groups are those to which an activated polyethylene glycol molecule may be bound. The amino acid residues having a free amino group may include lysine residues and the N-terminal amino acid residue. Those having a free carboxyl group may include aspartic acid residues, glutamic acid residues, and the C-terminal amino acid residue. Sulfhydryl groups may also be used as a reactive group for attaching the polyethylene glycol molecule(s). For therapeutic purposes, attachment at an amino group, such as attachment at the N-terminus or lysine group is preferred. Attachment at residues important for receptor binding should be avoided if receptor binding is desired.

One may specifically desire an N-terminal chemically modified protein. Using polyethylene glycol as an illustration of the present compositions, one may select from a variety of

polyethylene glycol molecules (by molecular weight, branching, etc.), the proportion of polyethylene glycol molecules to protein (or peptide) molecules in the reaction mix, the type of pegylation reaction to be performed, and the method of obtaining the selected N-terminally pegylated protein. The method of obtaining the N-terminally pegylated preparation (i.e., separating this moiety from other monopegylated moieties if necessary) may be by purification of the N-terminally pegylated material from a population of pegylated protein molecules. Selective N-terminal chemical modification may be accomplished by reductive alkylation which exploits differential reactivity of different types of primary amino groups (lysine versus the N-terminal) available for derivatization in a particular protein. Under the appropriate reaction conditions, substantially selective derivatization of the protein at the N-terminus with a carbonyl group containing polymer is achieved. For example, one may selectively N-terminally pegylate the protein by performing the reaction at a pH which allows one to take advantage of the pKa differences between the e-amino group of the lysine residues and that of the a-amino group of the N-terminal residue of the protein. By such selective derivatization, attachment of a water soluble polymer to a protein is controlled: the conjugation with the polymer takes place predominantly at the N-terminus of the protein and no significant modification of other reactive groups, such as the lysine side chain amino groups, occurs. Using reductive alkylation, the water soluble polymer may be of the type described above, and should have a single reactive aldehyde for coupling to the protein. Polyethylene glycol propionaldehyde, containing a single reactive aldehyde, may be used.

The present invention contemplates use of derivatives which are prokaryote-expressed neurturin, or variants thereof, linked to at least one polyethylene glycol molecule, as well as use of neurturin, or variants thereof, attached to one or more polyethylene glycol molecules via an acyl or alkyl linkage.

Pegylation may be carried out by any of the pegylation reactions known in the art. See, for example: Focus on Growth Factors, 3 (2):4-10, 1992; EP 0 154 316, the disclosure of which is hereby incorporated by reference; EP 0 401 384; and the other publications cited herein that relate to pegylation. The pegylation may be carried out via an acylation reaction or an alkylation reaction with a reactive polyethylene glycol molecule (or an analogous reactive water-soluble polymer).

Pegylation by acylation generally involves reacting an active ester derivative of polyethylene glycol with the neurturin protein or variant. Any known or subsequently discovered reactive PEG molecule may be used to carry out the pegylation of neurturin protein or variant. A preferred activated PEG ester is PEG esterified to N-hydroxysuccinimide. As used herein, "acylation" is contemplated to include without limitation the following types of linkages between the therapeutic protein and a water soluble polymer such as PEG: amide, carbamate, urethane, and the like. See Bioconjugate Chem., 5:133-140, 1994. Reaction conditions may be selected from any of those known in the pegylation art or those subsequently developed, but should avoid conditions of temperature, solvent, and pH that would inactivate the neurturin or variant to be modified.

Pegylation by acylation will generally result in a poly-pegylated neurturin protein or variant. Preferably, the connecting linkage will be an amide. Also preferably, the resulting product will be substantially only (e.g., > 95%) mono-, di- or tri-pegylated. However, some species with higher degrees of pegylation may be formed in amounts depending on the specific reaction conditions used. If desired, more purified pegylated species may be separated from the mixture, particularly unreacted species, by standard purification techniques, including, among others, dialysis, salting-out, ultrafiltration, ion-exchange chromatography, gel filtration chromatography and electrophoresis.

Pegylation by alkylation generally involves reacting a terminal aldehyde derivative of PEG with the neurturin protein or variant in the presence of a reducing agent. Pegylation by alkylation can also result in poly-pegylated neurturin protein or variant. In addition, one can

manipulate the reaction conditions to favor pegylation substantially only at the a-amino group of the N-terminus of the neurturin protein or variant (i.e., a mono-pegylated protein). In either case of monopegylation or polypegylation, the PEG groups are preferably attached to the protein via a -CH2-NH- group. With particular reference to the -CH2- group, this type of linkage is referred to herein as an "alkyl" linkage.

Derivatization via reductive alkylation to produce a monopegylated product exploits differential reactivity of different types of primary amino groups (lysine versus the N-terminal) available for derivatization. The reaction is performed at a pH which allows one to take advantage of the pKa differences between the e-amino groups of the lysine residues and that of the a-amino group of the N-terminal residue of the protein. By such selective derivatization, attachment of a water soluble polymer that contains a reactive group such as an aldehyde, to a protein is controlled: the conjugation with the polymer takes place predominantly at the N-terminus of the protein and no significant modification of other reactive groups, such as the lysine side chain amino groups, occurs. In one important aspect, the present invention contemplates use of a substantially homogeneous preparation of monopolymer/neurturin protein (or variant) conjugate molecules (meaning neurturin protein or variant to which a polymer molecule has been attached substantially only (i.e., > 95%) in a single location). More specifically, if polyethylene glycol is used, the present invention also encompasses use of pegylated neurturin protein or variant lacking possibly antigenic linking groups, and having the polyethylene glycol molecule directly coupled to the neurturin protein or variant.

Thus, it is contemplated that neurturin protein products to be used in accordance with the present invention may include pegylated neurturin protein or variants, wherein the PEG group(s) is (are) attached via acyl or alkyl groups. As discussed above, such products may be mono-pegylated or poly-pegylated (e.g., containing 2-6, and preferably 2-5, PEG groups). The PEG groups are generally attached to the protein at the a- or e-amino groups of amino acids, but it is also contemplated that the PEG groups could be attached to any amino group attached to the protein, which is sufficiently reactive to become attached to a PEG group under suitable reaction conditions.

The polymer molecules used in both the acylation and alkylation approaches may be selected from among water soluble polymers as described above. The polymer selected should be modified to have a single reactive group, such as an active ester for acylation or an aldehyde for alkylation, preferably, so that the degree of polymerization may be controlled as provided for in the present methods. An exemplary reactive PEG aldehyde is polyethylene glycol propionaldehyde, which is water stable, or mono C1-C10 alkoxy or aryloxy derivatives thereof (see, U.S. Pat. No. 5,252,714). The polymer may be branched or unbranched. For the acylation reactions, the polymer(s) selected should have a single reactive ester group. For the present reductive alkylation, the polymer(s) selected should have a single reactive aldehyde group. Generally, the water soluble polymer will not be selected from naturally-occurring glycosyl residues since these are usually made more conveniently by mammalian recombinant expression systems. The polymer may be of any molecular weight, and may be branched or unbranched.

A particularly preferred water-soluble polymer for use herein is polyethylene glycol. As used herein, polyethylene glycol is meant to encompass any of the forms of PEG that have been used to derivatize other proteins, such as mono-(C1-C10) alkoxy- or aryloxy-polyethylene glycol.

In general, chemical derivatization may be performed under any suitable condition used to react a biologically active substance with an activated polymer molecule. Methods for preparing a pegylated neurturin protein product will generally comprise the steps of (a) reacting a neurturin protein product with polyethylene glycol (such as a reactive ester or aldehyde derivative of PEG) under conditions whereby the protein becomes attached to one

or more PEG groups, and (b) obtaining the reaction product(s). In general, the optimal reaction conditions for the acylation reactions will be determined case-by-case based on known parameters and the desired result. For example, the larger the ratio of PEG:protein, the greater the percentage of poly-pegylated product.

Reductive alkylation to produce a substantially homogeneous population of a mono-polymer/neurturin protein product conjugate molecule will generally comprise the steps of: (a) reacting a neurturin protein product with a reactive PEG molecule under reductive alkylation conditions, at a pH suitable to permit selective modification of the a-amino group at the amino terminus of the neurturin protein product; and (b) obtaining the reaction product(s). ·

For a substantially homogeneous population of mono-polymer/neurturin protein product conjugate molecules, the reductive alkylation reaction conditions are those which permit the selective attachment of the water soluble polymer moiety to the N-terminus of neurturin protein product. Such reaction conditions generally provide for pKa differences between the lysine amino groups and the a-amino group at the N-terminus (the pKa being the pH at which 50% of the amino groups are protonated and 50% are not). The pH also affects the ratio of polymer to protein to be used. In general, if the pH is lower, a larger excess of polymer to protein will be desired (i.e., the less reactive the N-terminal a-amino group, the more polymer needed to achieve optimal conditions). If the pH is higher, the polymer:protein ratio need not be as large (i.e., more reactive groups are available, so fewer polymer molecules are needed). For purposes of the present invention, the pH will generally fall within the range of 3-9, preferably 3-6.

Another important consideration is the molecular weight of the polymer. In general, the higher the molecular weight of the polymer, the fewer polymer molecules may be attached to the protein. Similarly, branching of the polymer should be taken into account when optimizing these parameters. Generally, the higher the molecular weight (or the more branches) the higher the polymer:protein ratio. In general, for the pegylation reactions contemplated herein, the preferred average molecular weight is about 2 kDa to about 100 kDa. The preferred average molecular weight is about 5 kDa to about 50 kDa, particularly preferably about 12 kDa to about 25 kDa. The ratio of water-soluble polymer to neurturin protein product will generally range from 1:1 to 100:1, preferably (for polypegylation) 1:1 to 20:1 and (for monopegylation) 1:1 to 5:1.

Using the conditions indicated above, reductive alkylation will provide for selective attachment of the polymer to any neurturin protein product having an a-amino group at the amino terminus, and provide for a substantially homogenous preparation of monopolymer/neurturin protein product conjugate. The term "monopolymer/neurturin protein product conjugate" is used herein to mean a composition comprised of a single polymer molecule attached to a molecule of a neurturin protein product. The monopolymer/neurturin protein product conjugate preferably will have a polymer molecule located at the N-terminus, but not on lysine amino side groups. The preparation will preferably be greater than 90% monopolymer/neurturin protein product conjugate, and more preferably greater than 95% monopolymer/neurturin protein product conjugate, with the remainder of observable molecules being unreacted (i.e., protein lacking the polymer moiety).

For the present reductive alkylation, the reducing agent should be stable in aqueous solution and preferably be able to reduce only the Schiff base formed in the initial process of reductive alkylation. Preferred reducing agents may be selected from sodium borohydride, sodium cyanoborohydride, dimethylamine borane, trimethylamine borane and pyridine borane. A particularly preferred reducing agent is sodium cyanoborohydride. Other reaction parameters, such as solvent, reaction times, temperatures, etc., and means of purification of products, can be determined case-by-case based on the published information relating to

derivatization of proteins with water soluble polymers (see the publications cited herein).

C. Neurturin Protein Product Pharmaceutical Compositions

Neurturin protein product pharmaceutical compositions typically include a therapeutically effective amount of a neurturin protein product in admixture with one or more pharmaceutically and physiologically acceptable formulation materials selected for suitability with the mode of administration. Suitable formulation materials include, but are not limited to, antioxidants, preservatives, coloring, flavoring and diluting agents, emulsifying agents, suspending agents, solvents, fillers, bulking agents, buffers, delivery vehicles, diluents, excipients and/or pharmaceutical adjuvants. For example, a suitable vehicle may be water for injection, physiological saline solution, or artificial perilymph, possibly supplemented with other materials common in compositions for parenteral administration. Neutral buffered saline or saline mixed with serum albumin are further exemplary vehicles.

The primary solvent in a vehicle may be either aqueous or non-aqueous in nature. In addition, the vehicle may contain other pharmaceutically-acceptable excipients for modifying or maintaining the pH, osmolarity, viscosity, clarity, color, sterility, stability, rate of dissolution, or odor of the formulation. Similarly, the vehicle may contain still other pharmaceutically-acceptable excipients for modifying or maintaining the rate of release of neurturin protein product, or for promoting the absorption or penetration of neurturin protein product across the tympanic membrane. Such excipients are those substances usually and customarily employed to formulate dosages for middle-ear administration in either unit dose or multi-dose form.

Once the therapeutic composition has been formulated, it may be stored in sterile vials as a solution, suspension, gel, emulsion, solid, or dehydrated or lyophilized powder. Such formulations may be stored either in a ready to use form or in a form, e.g., lyophilized, requiring reconstitution prior to administration.

The optimal pharmaceutical formulations will be determined by one skilled in the art depending upon considerations such as the route of administration and desired dosage. See for example, Remington's Pharmaceutical Sciences, 18th Ed. (1990, Mack Publishing Co., Easton, Pa. 18042) pages 1435-1712, the disclosure of which is hereby incorporated by reference. Such formulations may influence the physical state, stability, rate of in vivo release, and rate of in vivo clearance of the present neurturin proteins, variants and derivatives.

Other effective administration forms, such as middle-ear slow-release formulations, inhalant mists, or orally active formulations are also envisioned. For example, in a sustained release formulation, the neurturin protein product may be bound to or incorporated into particulate preparations of polymeric compounds (such as polylactic acid, polyglycolic acid, etc.) or liposomes. Hylauronic acid may also be used, and this may have the effect of promoting sustained duration in the circulation.

Suitable biodegradable sustained release matrices include gelatin and polymers of, e.g., lactic acid, or collagens, including modified collagens such as atelocollagen, methylated collagen, or succinylated collagen. See, e.g., European Patent Application Publication No. EP 412 554 A2 published Feb. 13, 1991. Other suitable sustained release matrices include copolymers of L-glutamic acid and gamma ethyl-L-glutamate, poly (2-hydroxyethyl-methacrylate), ethylene vinyl acetate, poly-D-(-)-3-hydroxybutrrc acid, other polyesters, hyaluronic acid, or liposomes. The controlled release matrix may be prepared by mixing a GDNF solution or gel with the biodegradable matrix carrier, followed by concentrating and drying the mixture.

It is contemplated that a controlled release composition may be prepared in which the protein is dispersed in preformed porous polymeric microparticles. See PCT Application

Publication No. WO 93/15722, published Aug. 19, 1993. The microparticles may be prepared from any suitable polymeric material, such as polyesters, polyamides, polyanhydrides, or polyacrylates, and preferably is a biodegradable polymer, such as polylactic acid, poly-glycolic acid, a copolymer of lactic acid and glycolic acid, or poly[1,3-bis(p-carboxyphenoxy) propane-co-sebacic acid]. The microparticles, which are generally 50 to 400 microns in diameter and are permeated with a network of pores ranging from 0.01 to 1 microns, are loaded with protein by equilibrating them in a suspension or solution of protein. Vacuum or pressure may be applied to facilitate migration of the drug into the microparticles. The microparticles may be dried in air, under vacuum, by controlled evaporative drying, by a flowing inert gas, by freeze drying, or other techniques, and then further processed into desired compositions for injection or implantation.

The neurturin protein product pharmaceutical composition also may be formulated for middle-ear administration, e.g., by tympanic membrane infusion or injection, and may also include slow-release or sustained circulation formulations. Such middle-ear administered therapeutic compositions are typically in the form of a pyrogen-free, middle-ear acceptable aqueous solution comprising the neurturin protein product in a pharmaceutically acceptable vehicle. One preferred vehicle is sterile distilled water.

It is also contemplated that certain formulations containing neurturin protein product may be administered orally. A neurturin protein product which is administered in this fashion may be formulated as an elixir, tablet, capsule or gel and may be formulated with or without those carriers customarily used in the compounding of solid dosage forms. The capsule may be designed to release the active portion of the formulation at the point in the gastrointestinal tract when bioavailability is maximized and pre-systemic degradation is minimized. Additional excipients may be included to facilitate absorption of neurturin protein product. Diluents, flavorings, low melting point waxes, vegetable oils, lubricants, suspending agents, tablet disintegrating agents, and binders may also be employed.

The formulation of topical ear preparations, including middle-ear solutions, suspensions and ointments is well known to those skilled in the art (see Remington's Pharmaceutical Sciences, 18th Edition, Chapter 86, pages 1581-1592, Mack Publishing Company, 1990). Other modes of administration are available, including injections to the middle ear, and methods and means for producing middle-ear preparations suitable for such modes of administration are also well known.

As used in this application, "middle-ear" refers to the space between the tympanic membrane and the inner ear. This location is external to all inner ear tissue and an invasive procedure might not be required to access this region if a formulation is developed so that the neurturin will penetrate through the tympanic membrane. Alternatively, the material may be introduced to the middle ear by injection through the tympanic membrane or, in case repeated administrations are needed, a hole will be made in the tympanic membrane. Examples of such systems include inserts and "topically" applied drops, gels or ointments which may be used to deliver therapeutic material to these regions. An opening in the tymapanic membrane is a very frequent procedure done on a office-visit basis, in cases such as infections of the middle ear (usually in children). The opening closes spontaneously after a few days.

In the presently described use of neurturin protein product in the treatment of inner ear disease or injury it is also advantageous that a topically applied formulation include an agent to promote the penetration or transport of the therapeutic agent into the middle and inner ear. Such agents are known in the art. For example, Ke et al., U.S. Pat. No. 5,221,696 disclose the use of materials to enhance the penetration of ophthalmic preparations through the cornea.

Inner-ear systems are those systems which are suitable for use in any tissue compartment

within, between or around the tissue layers of the inner-ear, such as the cochlea and vestibular organ. These locations include the different structures of the cochlea such as the stria vascularis, Reissner's membrane, organ of Corti, spiral ligament and the cochlear neurons. An invasive procedure might not be required to access those structures since it has been shown that proteins do penetrate the membrane of the round window into the perilymph of the inner ear.

A particularly suitable vehicle for introducing neurturin into the inner ear by penetration through the round window membrane is artificial perilymph. This solution consists of 10.00 mM D-glucose, 1.5 mM CaCl, 1.5 mM MgCl in a 1.0% solution of Dulbeco's phosphate-buffered saline in deionized water at 280-300 mOsm and pH of 7.2. Yet another preparation may involve the formulation of the neurturin protein product with an agent, such as injectable microspheres or liposomes into the middle ear, that provides for the slow or sustained release of the protein which may then be delivered as a depot injection. Other suitable means for the inner-ear introduction of neurturin protein product includes, implantable drug delivery devices or which contain the neurturin protein product, and a cochlear-implant with a tunnel through, so neurturin can be continuously delivered through it to the inner ear.

The ear-treatment preparations of the present invention, particularly topical preparations, may include other components, for example middle-ear acceptable preservatives, tonicity agents, cosolvents, complexing agents, buffering agents, antimicrobials, antioxidants and surfactants, as are well known in the art. For example, suitable tonicity enhancing agents include alkali metal halides (preferably sodium or potassium chloride), mannitol, sorbitol and the like. Sufficient tonicity enhancing agent is advantageously added so that the formulation to be instilled into the ear is compatible with the osmolarity of the endo- and perilymph. Suitable preservatives include, but are not limited to, benzalkonium chloride, thimerosal, phenethyl alcohol, methylparaben, propylparaben, chlorhexidine, sodium benzoate, sorbic acid and the like. Hydrogen peroxide may also be used as preservative. Suitable cosolvents include, but are not limited to, alcohols, glycerin, glycerol, propylene glycol and polyethylene glycol. Suitable complexing agents include caffeine, polyvinylpyrrolidone, beta-cyclodextrin or hydroxypropyl-beta-cyclodextrin. Suitable antioxidants include sodium bisulfite and ascorbic acid. Suitable surfactants or wetting agents are for example sorbitan esters, polysorbates such as polysorbate 80, tromethamine, lecithin, cholesterol, tyloxapal and the like. The buffers can be conventional buffers such as acetate, borate, citrate, phosphate, bicarbonate, or Tris-HCl. Other stabilizing agents may be utilized, including proteins such as serum albumin, gelatin, or immunoglobulins, amino acids such as glycine, glutamate, aspartate, arginine, lysine or cysteine, and mono- and di-saccharides such as glucose, mannose or dextrin.

The formulation components are present in concentration that are acceptable to the middle or inner ear site of administration. For example, buffers are used to maintain the composition at physiological pH or at slightly lower pH, typically within a pH range of from about 5 to about 8.

Additional formulation components may include materials which provide for the prolonged residence of the middle ear administered therapeutic agent so as to maximize the topical contact and promote absorbtion through the round window membrane. Suitable materials include polymers or gel forming materials which provide for increased viscosity of the middle-ear preparation. The suitability of the formulations of the instant invention for controlled release (e.g., sustained and prolonged delivery) of an inner-ear treating agent can be determined by various procedures known in the art. Yet another ear preparation may involve an effective quantity of neurturin protein product in a mixture with non-toxic middle-ear treatment acceptable excipients which are suitable for the manufacture of tablets. By dissolving the tablets in sterile water, or other appropriate vehicle, middle-ear treatment solutions can be prepared in unit dose form. Suitable excipients include, but are not limited

to, inert diluents, such as calcium carbonate, sodium carbonate or bicarbonate, lactose, or calcium phosphate; or binding agents, such as starch, gelatin, or acacia.

Administration/Delivery of Neurturin Protein Product

The neurturin protein product may be administered parenterally via a subcutaneous, intramuscular, intravenous, intraarterial, intranasal, intrapulmonary, intraperitoneal, intraocular, transscleral, intravitreal, subretinal, intrathecal or intracerebral route. In appropriate circumstances, intralesional administration may be indicated, e.g. in irrigation fluid used to wash injured areas or implanted in injured areas with a suitable matrix. Alternatively, neurturin protein product may be administered orally, or into specific areas of the gastrointestinal tract, or via rectal, transdermal or topical routes.

For the treatment of inner-ear conditions, the neurturin protein product may be administered into the middle-ear (or directly into the inner-ear, especially in cases where an invasive procedure means is already in place), by topical application, inserts, injection, implants, cell therapy or gene therapy. For example, slow-releasing implants containing the neurotrophic factor embedded in a biodegradable polymer matrix can deliver neurturin protein product. A neurturin protein product may be administered extracerebrally in a form that has been modified chemically or packaged so that it passes the blood-brain barrier, or it may be administered in connection with one or more agents capable of promoting penetration of neurturin protein product across the barrier. Similarly, the neurturin protein product may be administered in the middle or inner ear, or it may be administered on top of the tympanic membrane in connection with one or more agents capable of promoting penetration or transport of neurturin protein product across the membranes of the ear. The frequency of dosing will depend on the pharmacokinetic parameters of the neurturin protein product as formulated, and the route of administration.

The specific dose may be calculated according to considerations of body weight, body surface area or organ size. Further refinement of the calculations necessary to determine the appropriate dosage for treatment involving each of the above mentioned formulations is routinely made by those of ordinary skill in the art and is within the ambit of tasks routinely performed, especially in light of the dosage information and assays disclosed herein. Appropriate dosages may be ascertained through use of the established assays for determining dosages utilized in conjunction with appropriate dose-response data. It will be appreciated by those skilled in the art that the dosage used in inner-ear administered formulations will be minuscule as compared to that used in a systemic injection or oral administration.

The final dosage regimen involved in a method for treating the above-described conditions will be determined by the attending physician, considering various factors which modify the action of drugs, e.g., the age, condition, body weight, sex and diet of the patient, the severity of any infection, time of administration and other clinical factors. As studies are conducted, further information will emerge regarding the appropriate dosage levels for the treatment of various diseases and conditions.

It is envisioned that the continuous administration or sustained delivery of a neurturin protein product may be advantageous for a given treatment. While continuous administration may be accomplished via a mechanical means, such as with an infusion pump, it is contemplated that other modes of continuous or near continuous administration may be practiced. For example, chemical derivatization or encapsulation may result in sustained release forms of the protein which have the effect of continuous presence, in predictable amounts, based on a determined dosage regimen. Thus, neurturin protein products include proteins derivatized or otherwise formulated to effectuate such continuous administration.

Neurturin protein product cell therapy, e.g., middle- or inner ear implantation of cells

producing neurturin protein product, is also contemplated. This embodiment would involve implanting into patients cells capable of synthesizing and secreting a biologically active form of neurturin protein product. Such neurturin protein product-producing cells may be cells that are natural producers of neurturin protein product or may be cells which are modified to express the protein. Such modified cells include recombinant cells whose ability to produce a neurturin protein product has been augmented by transformation with a gene encoding the desired neurturin protein product in a vector suitable for promoting its expression and secretion. In order to minimize a potential immunological reaction in patients being administered neurturin protein product of a foreign species, it is preferred that the natural cells producing neurturin protein product be of human origin and produce human neurturin protein product. Likewise, it is preferred that the recombinant cells producing neurturin protein product be transformed with an expression vector containing a gene encoding a human neurturin protein product. Implanted cells may be encapsulated to avoid infiltration of surrounding tissue. Human or non-human animal cells may be implanted in patients in biocompatible, semipermeable polymeric enclosures or membranes that allow release of neurturin protein product, but that prevent destruction of the cells by the patient's immune system or by other detrimental factors from the surrounding tissue. Such an implant, for example, may be attached to the round-window membrane of the middle-ear to produce and release neurturin protein product directly into the perilymph.

The methodology for the membrane encapsulation of living cells is familiar to those of ordinary skill in the art, and the preparation of the encapsulated cells and their implantation in patients may be accomplished without undue experimentation. See, e.g., U.S. Pat. Nos. 4,892,538, 5,011,472, and 5,106,627, each of which is specifically incorporated herein by reference. A system for encapsulating living cells is described in PCT Application WO 91/10425 of Aebischer et al., specifically incorporated herein by reference. See also, PCP Application WO 91/10470 of Aebischer et al., Winn et al., Exper. Neurol., 113 :322-329, 1991, Aebischer et al., Exper. Neurol., 111:269-275, 1991; Tresco et al., ASAIO, 38:17-23, 1992, each of which is specifically incorporated herein by reference. Techniques for formulating a variety of other sustained- or controlled-delivery means, such as liposome carriers, bio-erodible particles or beads and depot injections, are also known to those skilled in the art.

It is also contemplated that the patient's own cells may be transformed ex vivo to produce neurturin protein product and would be directly implanted without encapsulation. For example, organ of Corti supporting cells may be retrieved, the cells cultured and transformed with an appropriate vector and transplanted back into the patient's inner ear where they would produce and release the desired neurturin protein or neurturin protein variant.

Neurturin protein product gene therapy in vivo is also envisioned, by introducing the gene coding for neurturin protein product into targeted inner ear cells via local injection of a nucleic acid construct or other appropriate delivery vectors. (Hefti, J. Neurobiol., 25:1418-1435, 1994). For example, a nucleic acid sequence encoding a neurturin protein product may be contained in an adeno-associated virus vector or adenovirus vector for delivery to the inner ear cells. Alternative viral vectors include, but are not limited to, retrovirus, herpes simplex virus and papilloma virus vectors. Physical transfer, either in vivo or ex vivo as appropriate, may also be achieved by liposome-mediated transfer, direct injection (naked DNA), receptor-mediated transfer (ligand-DNA complex), electroporation, calcium phosphate precipitation or microparticle bombardment (gene gun).

It should be noted that the neurturin protein product formulations described herein may be used for veterinary as well as human applications and that the term "patient" should not be construed in a limiting manner. In the case of veterinary applications, the dosage ranges should be the same as specified above.

Polynucleotides Encoding Neurturin Protein Product

The present invention further provides novel polynucleotides which encode neurturin protein products. When used as a hybridization probe or amplification primer, the nucleic acid sequence will be substantially free from all other nucleic acid sequences. For use in recombinant protein expression, the nucleic acid sequence will generally be substantially free from nucleic acid sequences encoding other proteins, unless a fusion protein is intended. Based upon the present description and using the universal codon table, one of ordinary skill in the art can readily determine all of the nucleic acid sequences which encode the amino acid sequences of a neurturin protein product. It will also be appreciated by those skilled in the art that the novel polynucleotides which encode neurturin protein products include those nucleic acid sequences encoding variant proteins, whether man-made or naturally occurring.

Recombinant expression techniques, conducted in accordance with the descriptions set forth below, may be followed to produce these polynucleotides and express the various neurturin protein products. For example, by inserting a nucleic acid sequence which encodes a protein into an appropriate vector, one skilled in the art can readily produce large quantities of the desired nucleotide sequence. The sequences can then be used to generate detection probes or amplification primers. Alternatively, a polynucleotide encoding a neurturin protein product can be inserted into an expression vector. By introducing the expression vector into an appropriate host, the desired protein may be produced in large amounts.

As further described herein, there are numerous host/vector systems available for the propagation of nucleic acid sequences and/or the production of neurturin protein products. These include, but are not limited to, plasmid, viral and insertional vectors, and prokaryotic and eukaryotic hosts. One skilled in the art can adapt a host/vector system which is capable of propagating or expressing heterologous DNA to produce or express the sequences of the present invention.

By means of such recombinant techniques, the proteins of the present invention are readily produced in commercial quantities. Furthermore, it will be appreciated by those skilled in the art that, in view of the present disclosure, the novel nucleic acid sequences include degenerate nucleic acid sequences encoding the proteins specifically set forth in the Figures, variants of such proteins, and those nucleic acid sequences which hybridize, preferably under stringent hybridization conditions, to complements of these nucleic acid sequences (see, Maniatis et al., Molecular Cloning (A Laboratory Manual); Cold Spring Harbor Laboratory, pages 387 to 389, 1982.) Exemplary stringent hybridization conditions are hybridization in 4 x SSC at 62-67o C., followed by washing in 0.1 x SSC at 62-67o C. for approximately an hour. Alternatively, exemplary stringent hybridization conditions are hybridization in 45-55% formamide, 4 x SSC at 40-45o C. DNA sequences which hybridize to the complementary sequences for neurturin protein under relaxed hybridization conditions and which encode a neurturin protein of the present invention are also included herein. Examples of such relaxed stringency hybridization conditions are 4 x SSC at 45-55o C. or hybridization with 30-40% formamide at 40-45o C.

Also provided by the present invention are recombinant DNA constructs involving vector DNA together with the DNA sequence encoding a neurturin protein product. In such DNA constructs, the nucleic acid sequence encoding the protein (with or without signal peptides) is in operative association with a suitable expression control or regulatory sequence capable of directing the replication and/or expression of the protein in a selected host.

Recombinant Expression of a Neurturin Protein Product

Preparation of Polynucleotides Encoding Neurturin Protein Products

A nucleic acid sequence encoding a neurturin protein product, can readily be obtained in a variety of ways, including, without limitation, chemical synthesis, cDNA or genomic library

screening, expression library screening, and/or PCR amplification of cDNA. These methods and others useful for isolating such nucleic acid sequences are set forth, for example, by Sambrook et al. (Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1989), by Ausubel et al., eds (Current Protocols in Molecular Biology, Current Protocols Press, 1994), and by Berger and Kimmel (Methods in Enzymology: Guide to Molecular Cloning Techniques, vol. 152, Academic Press, Inc., San Diego, Calif., 1987).

Chemical synthesis of a nucleic acid sequence which encodes a neurturin protein product can also be accomplished using methods well known in the art, such as those set forth by Engels et al. (Angew. Chem. Intl. Ed., 28:716-734, 1989). These methods include, inter alia, the phosphotriester, phosphoramidite and H-phosphonate methods of nucleic acid sequence synthesis. The nucleic acid sequence encoding the protein will be several hundred base pairs (bp) or nucleotides in length. Large nucleic acid sequences, for example those larger than about 100 nucleotides in length, can be synthesized as several fragments. The fragments can then be ligated together to form a nucleic acid sequence encoding the protein. A preferred method is polymer-supported synthesis using standard phosphoramidite chemistry.

Alternatively, a suitable nucleic acid sequence may be obtained by screening an appropriate cDNA library (i.e., a library prepared from one or more tissue source(s) believed to express the protein) or a genomic library (a library prepared from total genomic DNA). The source of the cDNA library is typically a tissue from any species that is believed to express neurturin in reasonable quantities. The source of the genomic library may be any tissue or tissues from any mammalian or other species believed to harbor a gene encoding neurturin or a neurturin homologue. The library can be screened for the presence of the neurturin cDNA/gene using one or more nucleic acid probes (oligonucleotides, cDNA or genomic DNA fragments that possess an acceptable level of homology to the neurturin or neurturin homologue cDNA or gene to be cloned) that will hybridize selectively with neurturin or neurturin homologue cDNA (s) or gene(s) present in the library. The probes typically used for such library screening usually encode a small region of the neurturin DNA sequence from the same or a similar species as the species from which the library was prepared. Alternatively, the probes may be degenerate, as discussed herein.

Library screening is typically accomplished by annealing the oligonucleotide probe or cDNA to the clones in the library under conditions of stringency that prevent non-specific binding but permit binding of those clones that have a significant level of homology with the probe or primer. Typical hybridization and washing stringency conditions depend in part on the size (i.e., number of nucleotides in length) of the cDNA or oligonucleotide probe, and whether the probe is degenerate. The probability of obtaining a clone(s) is also considered in designing the hybridization solution (i.e., whether a cDNA or genomic library is being screened; if it is a cDNA library, the probability that the cDNA of interest is present at a high level).

Where DNA fragments (such as cDNAs) are used as probes, typical hybridization conditions include those as set forth in Ausubel et al., eds., supra. After hybridization, the blot containing the library is washed at a suitable stringency, depending on several factors such as probe size, expected homology of probe to clone, type of library being screened, number of clones being screened, and the like. Examples of stringent washing solutions (which are usually low in ionic strength and are used at relatively high temperatures) are as follows. One such stringent wash is 0.015 M NaCl, 0.005 M NaCitrate and 0.1% SDS at 55-65o C. Another such stringent buffer is 1 mM Na2EDTA, 40 mM NaHPO4, pH 7.2, and 1% SDS at about 40-50o C. One other stringent wash is 0.2 x SSC and 0.1% SDS at about 50-65o C.

There are also exemplary protocols for stringent washing conditions where oligonucleotide probes are used to screen cDNA or genomic libraries. For example, a first protocol uses 6 x SSC with 0.05 percent sodium pyrophosphate at a temperature of between about 35 and 62o C., depending on the length of the probe. For example, 14 base probes are washed at 35-40o

C., 17 base probes at 45-50o C., 20 base probes at 52-57o C., and 23 base probes at 57-63o C. The temperature can be increased 2-3o C. where the background non-specific binding appears high. A second protocol uses tetramethylammonium chloride (TMAC) for washing. One such stringent washing solution is 3 M TMAC, 50 mM Tris-HCl, pH 8.0, and 0.2% SDS.

Another suitable method for obtaining a nucleic acid sequence encoding a neurturin protein product is the polymerase chain reaction (PCR). In this method, poly(A) + RNA or total RNA is extracted from a tissue that expresses neurturin. cDNA is then prepared from the RNA using the enzyme reverse transcriptase. Two primers, typically complementary to two separate regions of the neurturin cDNA (oligonucleotides), are then added to the cDNA along with a polymerase such as Taq polymerase, and the polymerase amplifies the cDNA region between the two primers.

Where the method of choice for preparing the nucleic acid sequence encoding the desired neurturin protein product requires the use of oligonucleotide primers or probes (e.g., PCR, cDNA or genomic library screening), the oligonucleotide sequences selected as probes or primers should be of adequate length and sufficiently unambiguous so as to minimize the amount of non-specific binding that will occur during library screening or PCR amplification. The actual sequence of the probes or primers is usually based on conserved or highly homologous sequences or regions from the same or a similar gene from another organism. Optionally, the probes or primers can be fully or partially degenerate, i.e., contain a mixture of probes/primers, all encoding the same amino acid sequence, but using different codons to do so. An alternative to preparing degenerate probes is to place an inosine in some or all of those codon positions that vary by species. The oligonucleotide probes or primers may be prepared by chemical synthesis methods for DNA as described above.

Neurturin protein products based on these nucleic acid sequences, as well as mutant or variant sequences thereof, are also contemplated as within the scope of the present invention. As described above, a mutant or variant sequence is a sequence that contains one or more nucleotide substitutions, deletions, and/or insertions as compared to the wild type sequence and that results in the expression of amino acid sequence variations as compared to the wild type amino acid sequence. In some cases, naturally occurring neurturin amino acid mutants or variants may exist, due to the existence of natural allelic variation. Neurturin protein products based on such naturally occurring mutants or variants are also within the scope of the present invention. Preparation of synthetic mutant sequences is also well known in the art, and is described for example in Wells et al. (Gene, 34:315, 1985) and in Sambrook et al., supra.

Vectors

The cDNA or genomic DNA encoding a neurturin protein product is inserted into a vector for further cloning (amplification of the DNA) or for expression. Suitable vectors are commercially available, or the vector may be specially constructed. The selection or construction of the appropriate vector will depend on 1) whether it is to be used for DNA amplification or for DNA expression, 2) the size of the DNA to be inserted into the vector, and 3) the host cell (e.g., mammalian, insect, yeast, fungal, plant or bacterial cells) to be transformed with the vector. Each vector contains various components depending on its function (amplification of DNA or expression of DNA) and its compatibility with the intended host cell. The vector components generally include, but are not limited to, one or more of the following: a signal sequence, an origin of replication, one or more selection or marker genes, enhancer elements, promoters, a transcription termination sequence, and the like. These components or expression regulatory elements may be obtained from natural sources or synthesized by known procedures. The vectors of the present invention involve a nucleic acid sequence which encodes the neurturin protein product of interest operatively linked to one or more of the following expression control or regulatory sequences capable of directing, controlling or otherwise effecting the expression of the protein by a selected host cell.

Signal Sequence

The signal sequence may be a component of the vector, or it may be a part of the neurturin protein product DNA that is inserted into the vector. The neurturin DNA encodes a signal sequence at the amino terminus of the protein that is cleaved during post-translational processing of the protein to form the mature protein. Included within the scope of this invention are neurturin protein product polynucleotides with the native signal sequence and other pre-pro sequences as well as polynucleotides wherein the native signal sequence is deleted and replaced with a heterologous signal sequence. The heterologous signal sequence selected should be one that is recognized and processed, i.e., cleaved by a signal peptidase, by the host cell. For prokaryotic host cells that do not recognize and process the native neurturin signal sequence, the signal sequence is substituted by a prokaryotic signal sequence selected, for example, from the group of the alkaline phosphatase, penicillinase, or heat-stable enterotoxin II leaders. For yeast secretion, the native neurturin signal sequence may be substituted by the yeast invertase, alpha factor, or acid phosphatase leaders. In mammalian cell expression the native signal sequence is satisfactory, although other mammalian signal sequences may be suitable.

Origin of Replication

Expression and cloning vectors generally include a nucleic acid sequence that enables the vector to replicate in one or more selected host cells. In cloning vectors, this sequence is typically one that enables the vector to replicate independently of the host chromosomal DNA, and includes origins of replication or autonomously replicating sequences. Such sequences are well known for a variety of bacteria, yeasts, and viruses. The origin of replication from the plasmid pBR322 is suitable for most Gram-negative bacteria and various origins (e.g., SV40, polyoma, adenovirus, VSV or BPV) are useful for cloning vectors in mammalian cells. Generally, the origin of replication component is not needed for mammalian expression vectors (for example, the SV40 origin is often used only because it contains the early promoter).

Selection Gene

The expression and cloning vectors typically contain a selection gene. This gene encodes a "marker" protein necessary for the survival or growth of the transformed host cells when grown in a selective culture medium. Host cells that were not transformed with the vector will not contain the selection gene, and therefore, they will not survive in the culture medium. Typical selection genes encode proteins that (a) confer resistance to antibiotics or other toxins, e.g., ampicillin, neomycin, methotrexate, or tetracycline; (b) complement auxotrophic deficiencies; or (c) supply critical nutrients not available from the culture medium.

Other selection genes may be used to amplify the gene which will be expressed. Amplification is the process wherein genes which are in greater demand for the production of a protein critical for growth are reiterated in tandem within the chromosomes of successive generations of recombinant cells. Examples of suitable selectable markers for mammalian cells include dihydrofolate reductase (DHFR) and thymidine kinase. The mammalian cell transformants are placed under selection pressure which only the transformants are uniquely adapted to survive by virtue of the marker present in the vector. Selection pressure is imposed by culturing the transformed cells under conditions in which the concentration of selection agent in the medium is successively changed, thereby leading to amplification of both the selection gene and the DNA that encodes a neurturin protein product. As a result, increased quantities of the neurturin protein product are synthesized from the amplified DNA.

For example, cells transformed with the DHFR selection gene are first identified by culturing

all of the transformants in a culture medium that contains methotrexate, a competitive antagonist of DHFR. An appropriate host cell when wild-type DHFR is used is the Chinese hamster ovary cell line deficient in DHFR activity (see, for example, Urlaub and Chasin, Proc. Natl. Acad. Sci., USA 77(7): 4216-4220 (1980)). The transformed cells are then exposed to increased levels of methotrexate. This leads to the synthesis of multiple copies of the DHFR gene, and, concomitantly, multiple copies of other DNA present in the expression vector, such as the DNA encoding a neurturin protein.

Promoter

The expression and cloning vectors of the present invention will typically contain a promoter that is recognized by the host organism and operably linked to the nucleic acid sequence encoding the neurturin protein product. Promoters are untranslated sequences located upstream (5') to the start codon of a structural gene (generally within about 100 to 1000 bp) that control the transcription and translation of a particular nucleic acid sequence. Promoters are conventionally grouped into one of two classes, inducible promoters and constitutive promoters. Inducible promoters initiate increased levels of transcription from DNA under their control in response to some change in culture conditions, such as the presence or absence of a nutrient or a change in temperature. A large number of promoters, recognized by a variety of potential host cells, are well known. These promoters are operably linked to the DNA encoding neurturin by removing the promoter from the source DNA by restriction enzyme digestion and inserting the desired promoter sequence into the vector. The native neurturin promoter sequence may be used to direct amplification and/or expression of neurturin DNA. A heterologous promoter is preferred, however, if it permits greater transcription and higher yields of the expressed protein as compared to the native promoter, and if it is compatible with the host cell system that has been selected for use.

Promoters suitable for use with prokaryotic hosts include the betalactamase and lactose promoter systems; alkaline phosphatase, a tryptophan (trp) promoter system; and hybrid promoters such as the tac promoter. Other known bacterial promoters are also suitable. Their nucleotide sequences have been published, thereby enabling one skilled in the art to ligate them to the desired DNA sequence(s), using linkers or adaptors as needed to supply any required restriction sites.

Suitable promoting sequences for use with yeast hosts are also well known in the art. Yeast enhancers are advantageously used with yeast promoters. Suitable promoters for use with mammalian host cells are well known and include those obtained from the genomes of viruses such as polyoma virus, fowlpox virus, adenovirus (such as Adenovirus 2), bovine papilloma virus, avian sarcoma virus, cytomegalovirus, a retrovirus, hepatitis-B virus and most preferably Simian Virus 40 (SV40). Other suitable mammalian promoters include heterologous mammalian promoters, e.g., heat-shock promoters and the actin promoter. A currently used promoter in the production of proteins in CHO cells is SRa. See Takebe et al., Mol. Cell. Biol. 8(1): 466-472 (1988).

Enhancer Element

An enhancer sequence may be inserted into the vector to increase the transcription of a DNA sequence encoding a protein of the present invention by higher eukaryotes. Enhancers are cis-acting elements of DNA, usually about from 10-300 bp in length, that act on the promoter to increase its transcription. Enhancers are relatively orientation and position independent. They have been found 5' and 3' to the transcription unit. Several enhancer sequences available from mammalian genes are known (e.g., globin, elastase, albumin, alpha-fetoprotein and insulin). Typically, however, an enhancer from a virus will be used. The SV40 enhancer, the cytomegalovirus early promoter enhancer, the polyoma enhancer, and adenovirus enhancers are exemplary enhancing elements for the activation of eukaryotic promoters. While an enhancer may be spliced into the vector at a position 5' or 3' to

neurturin DNA, it is typically located at a site 5' from the promoter.

Transcription Termination

Expression vectors used in eukaryotic host cells (yeast, fungi, insect, plant, animal, human, or nucleated cells from other multicellular organisms) will also contain sequences necessary for the termination of transcription and for stabilizing the mRNA. Such sequences are commonly available from the 5' and occasionally 3' untranslated regions of eukaryotic DNAs or cDNAs. These regions contain nucleotide segments transcribed as polyadenylated fragments in the untranslated portion of the mRNA encoding the protein.

The construction of suitable vectors containing one or more of the above-listed components together with the desired neurturin protein product coding sequence is accomplished by standard ligation techniques. Isolated plasmids or DNA fragments are cleaved, tailored, and religated in the desired order to generate the plasmids required. To confirm that the correct sequences have been constructed, the ligation mixtures may be used to transform E. coli, and successful transformants may be selected by known techniques, such as ampicillin or tetracycline resistance as described above. Plasmids from the transformants are then prepared, analyzed by restriction endonuclease digestion, and/or sequenced to confirm the presence of the desired construct.

Vectors that provide for the transient expression of DNA encoding a neurturin protein product in mammalian cells may also be used. In general, transient expression involves the use of an expression vector that is able to replicate efficiently in a host cell, such that the host cell accumulates many copies of the expression vector and, in turn, synthesizes high levels of the desired protein encoded by the expression vector. Transient expression systems, comprising a suitable expression vector and a host cell, allow for the convenient positive identification of proteins encoded by cloned DNAs, as well as for the rapid screening of such proteins for desired biological or physiological properties. Thus, transient expression systems are particularly useful in identifying variants of the protein.

Selection and Transformation of Host Cells

Host cells (e.g., bacterial, mammalian, insect, yeast, or plant cells) transformed with nucleic acid sequences for use in expressing a recombinant neurturin protein are also provided by the present invention. The transformed host cell is cultured under appropriate conditions permitting the expression of the nucleic acid sequence. The selection of suitable host cells and methods for transformation, culture, amplification, screening and product production and purification are well known in the art. See for example, Gething and Sambrook, Nature 293: 620-625 (1981), or alternatively, Kaufman et al., Mol. Cell. Biol., 5 (7): 1750-1759 (1985) or Howley et al., U.S. Pat. No. 4,419,446. The transformed host cell is cultured in a suitable medium, and the expressed factor is then optionally recovered, isolated and purified from the culture medium (or from the cell, if expressed intracellularly) by an appropriate means known to those skilled in the art.

Suitable host cells for cloning or expressing the vectors herein are the prokaryote, yeast, or higher eukaryote cells as described above. Prokaryotic host cells include, but are not limited to, eubacteria, such as Gram-negative or Gram-positive organisms, for example, E. coli, Bacilli such as B. subtilis, Pseudomonas species such as P. aeruginosa, Salmonella typhimurium, or Serratia marcescans. Alternatively, in vitro methods of cloning, e.g., PCR or other nucleic acid polymerase reactions, are suitable.

In addition to prokaryotic host cells, eukaryotic microbes such as filamentous fungi or yeast may be suitable hosts for the expression of neurturin protein products. Saccharomyces cerevisiae, or common baker's yeast, is the most commonly used among lower eukaryotic host microorganisms, but a number of other genera, species, and strains are well known and

commonly available.

Suitable host cells for the expression of glycosylated neurturin protein products are derived from multicellular organisms. Such host cells are capable of complex processing and glycosylation activities. In principle, any higher eukaryotic cell culture might be used, whether such culture involves vertebrate or invertebrate cells, including plant and insect cells. Vertebrate cells are generally used as the propagation of vertebrate cells in culture (tissue culture) is a well known procedure. Examples of useful mammalian host cell lines include, but are not limited to, monkey kidney CV1 line transformed by SV40 (COS-7), human embryonic kidney line (293 or 293 cells subcloned for growth in suspension culture), baby hamster kidney cells, and Chinese hamster ovary cells. Other suitable mammalian cell lines include but are not limited to, HeLa, mouse L-929 cells, 3T3 lines derived from Swiss, Balb-c or NIH mice, BHK or HaK hamster cell lines.

Similarly useful as host cells suitable for the present invention are bacterial cells. For example, the various strains of E. coli (e.g., HB101, DH5a, DH10, and MC1061) are well-known as host cells in the field of biotechnology. Various strains of Streptomyces spp. and the like may also be employed. Presently preferred host cells for producing neurturin proteins are bacterial cells (e.g., Escherichia coli) and mammalian cells (such as Chinese hamster ovary cells, COS cells, etc.)

The host cells are transfected and preferably transformed with the above-described expression or cloning vectors and cultured in a conventional nutrient medium. The medium may be modified as appropriate for inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences. Transfection and transformation are performed using standard techniques which are well known to those skilled in the art and which are selected as appropriate to the host cells involved. For example, for mammalian cells without cell walls, the calcium phosphate precipitation method may be used. Electroporation, micro injection and other known techniques may also be used.

Culturing the Host Cells

Transformed cells used to produce proteins of the present invention are cultured in suitable media. The media may be supplemented as necessary with hormones and/or other growth factors (such as insulin, transferrin, or epidermal growth factor), salts (such as sodium chloride, calcium, magnesium, and phosphate), buffers (such as HEPES), nucleosides (such as adenosine and thymidine), antibiotics (such as gentamicin), trace elements (defined as inorganic compounds usually present at final concentrations in the micromolar range), and glucose or other energy source. Other supplements may also be included, at appropriate concentrations, as will be appreciated by those skilled in the art. Suitable culture conditions, such as temperature, pH, and the like, are also well known to those skilled in the art for use with the selected host cells.

It is also possible that neurturin protein product may be produced by homologous recombination, or with recombinant production methods utilizing control elements introduced into cells already containing DNA encoding neurturin or GDNF. Homologous recombination is a technique originally developed for targeting genes to induce or correct mutations in transcriptionally active genes (Kucherlapati, Prog. in Nucl. Acid Res. and Mol. Biol. 36:301 (1989)). The basic technique was developed as a method for introducing specific mutations into specific regions of the mammalian genome (Thomas et al., Cell. 44:419-428, 1986; Thomas and Capecchi, Cell. 51:503-512, 1987; Doetschman et al., Proc. Natl. Acad. Sci. 85:8583-8587, 1988) or to correct specific mutations within defective genes (Doetschman et al., Nature. 330:576-578, 1987). Exemplary homologous recombination techniques are described in U.S. Pat. No. 5,272,071 (EP 91 90 3051, EP Publication No. 505 500; PCT/US90/07642, International Publication No. WO 91/09955) the disclosure of which is hereby incorporated by reference.

Through homologous recombination, the DNA sequence to be inserted into the genome can be directed to a specific region of the gene of interest by attaching it to targeting DNA. The targeting DNA is DNA that is complementary (homologous) to a region of the genomic DNA. Small pieces of targeting DNA that are complementary to a specific region of the genome are put in contact with the parental strand during the DNA replication process. It is a general property of DNA that has been inserted into a cell to hybridize and therefore recombine with other pieces of endogenous DNA through shared homologous regions. If this complementary strand is attached to an oligonucleotide that contains a mutation or a different sequence of DNA, it too is incorporated into the newly synthesized strand as a result of the recombination. As a result of the proofreading function, it is possible for the new sequence of DNA to serve as the template. Thus, the transferred DNA is incorporated into the genome.

If the sequence of a particular gene is known, such as the nucleic acid sequence of a neurturin protein product, the pre-pro sequence or expression control sequence, a piece of DNA that is complementary to a selected region of the gene can be synthesized or otherwise obtained, such as by appropriate restriction of the native DNA at specific recognition sites bounding the region of interest. This piece serves as a targeting sequence upon insertion into the cell and will hybridize to its homologous region within the genome. If this hybridization occurs during DNA replication, this piece of DNA, and any additional sequence attached thereto, will act as an Okazaki fragment and will be backstitched into the newly synthesized daughter strand of DNA.

In the present invention, attached to these pieces of targeting DNA are regions of DNA which may interact with the expression of a neurturin protein product. For example, a promoter/enhancer element, a suppresser, or an exogenous transcription modulatory element is inserted in the genome of the intended host cell in proximity and orientation sufficient to influence the transcription of DNA encoding the neurturin protein product. The control element does not encode neurturin, but instead controls a portion of the DNA present in the host cell genome. Thus, the expression of the protein may be achieved not by transfection of DNA that encodes the neurturin protein product gene itself, but rather by the use of targeting DNA (containing regions of homology with the endogenous gene of interest) coupled with DNA regulatory segments that provide the endogenous gene sequence with recognizable signals for transcription of a neurturin protein product. In accordance with the present invention, homologous recombination methods may also be used to modify a cell that contains a normally transcriptionally silent neurturin protein product gene to produce a cell which expresses neurturin protein product.

Other aspects and advantages of the present invention will be understood upon consideration of the following illustrative examples. Example 1 addresses the effects of neurturin protein product administration on hair cells in a Cochlea explant culture system. Example 3 addresses the effects of neurturin protein product administration on inner ear auditory neurons (spiral ganglion neurons), in a dissociated cell culture generated from cochlea.

EXAMPLES

Example 1

Neurturin Protein Product Protects Cochlear Hair Cells Against Ototoxicity

Materials

The materials used in the following Example were obtained as follows.

Organ of Corti Dissecting Solution

Dulbecco's Phosphate Buffered Saline (PBS; 1 x , without calcium chloride, without magnesium chloride. Cat. #14190-136, Gibco BRL), containing 1.5 g/L D-Glucose (Dextrose. Cat. #15023-021, Gibco BRL).

Organ of Corti Explant Culture Medium

1. High glucose Dulbecco's Modified Eagle Medium (DMEM; 1 x , with L-glutamine, without Sodium Pyruvate. Cat. #1 1965-084, Gibco BRL)

2. 0.15 g/100 ml of D-Glucose (Dextrose. Cat. #15023-021, Gibco BRL)

3. 1% N-2 Supplement (100X, Cat. #17502-030, Gibco BRL)

4. 100 Units/ml of Penicillin G, Potassium (Penicillin; Cat. # 21840-020, Gibco BRL)

Methods

Preparation of Medium

DMEM was supplemented with 1% N-2 supplement, and D-glucose was added to a final concentration of 1.5 g/L. Penicillin was added at 100 Units/ml. After mixing, the medium was filtered and kept at 4o C. The medium was prepared fresh just before use in order to minimize inter-experimental variations. Plastic pipettes and containers were used throughout to minimize protein adsorption.

Neurturin Protein Product Solutions

Human recombinant neurturin protein products were prepared as 1 mg/ml solutions in D-PBS (phosphate buffered saline prepared with distilled water) containing five percent bovine serum albumin. The solutions were stored at - 85o C. in aliquots. Serial dilutions (0.1; 1; 10; 50; 100 ng/ml in normal culture medium) were prepared in 96 microplates. Ten microliters of ten-fold concentrated neurturin protein product solutions were added to Organ of Corti explant cultures medium containing ototoxins or not (control)(90 mu l). Control cultures received normal medium(10 mu l). The neurturin protein product treatments were initiated on day of plating. On the second day, media were exchanged into media containing the ototoxins alone, together with neurturin or without both (control).

Dissecting Tools and Culture Dishes

1. The 4" and 5" dissecting forceps and 4" dissecting scissors were from Roboz Surgical, Washington, D.C.

2. Falcon sterile 96-well microplates (Flat Bottom. Cat. #3072), tissue culture plastic ware and polypropylene centrifuge tubes were from Beckton-Dickinson, Lincoln Park, N.J.

Ototoxins and Related Reagents

1. Neomycin solution (Cat. #N1142, Sigma. St. Louis, Mo.), used at a final concentration of 0.6 mM (a fresh solution was made for each experiment by adding 90 mu l of 1 mg/ml neomycin and to 1410 mu l medium).

2. Cisplatin (Platinol-AQ. Cat. #NDC 0015-3220-22, Bristol-Myers Squibb Laboratories, Princeton, N.J.). Used at a final concentration of 35 mu g/ml (a fresh solution was prepared

for each experiment by adding 52.5 mu l of 1 mg/ml cisplatin to 1447.5 mu l medium).

3. Triton X-100 (t-Octylphenoxypoly-ethoxyethanol. Cat. #X-100, Sigma. St. Louis, Mo.)

4. Phalloidin (FITC Labeled. Cat. #P-5282, Sigma. St. Louis, Mo.)

5. Vectashield (Mounting Medium, Cat. #H-1000, Vector, Burlingame, Calif.)

Preparation of Rat Organ of Corti Explant

Organ of Corti explants were obtained from P3-P4 Wistar rats. Rats were decapitated, the lower jaw was cut out and skin removed. The temporal bone was collected in dissection solution, the otic capsule exposed and the bony-cartilaginous cochlear capsule was carefully separated from the temporal bone. Freed cochlea were transferred to another Petri dish with dissection solution for further dissection. Intact organs of Corti were obtained by using a fine forceps to hold central VIII nerve tissue and remove it out, then the stria vascular membrane was carefully stripped off, starting from the apex or base. The organ of Corti was then transferred to a 35-mm diameter Petri dish containing cold PBS supplemented with glucose and ready to be cultured.

Cochlea Explant Culture Procedure

Cochlea explants were cultured in uncoated 96 well microplates. A single organ of Corti was placed in a well and was kept floating in the medium.

Explants were kept in normal medium for 24 hours (90 mu l/well). Neurturin protein solution (10 mu l) was added to the 'treated' cultures, and 10 mu l of medium were added to controls. After 24 hours of incubation, the media were changed and the explants were exposed to ototoxin-containing medium (90 mu l), with neurturin protein solution (10 mu l) or without (control). The cultures were incubated for an additional 3 days. The explants were then fixed with 4% paraformaldehyde in 0.1 M D-PBS for 30 minutes at room temperature and processed for immunostaining.

FITC-Phalloidin Staining of Hair Cells

To identify and count hair cells in the organ of Corti, a direct immunostaining method was used to label the actin present naturally in the stereocilia bundles of the hair cells. The explants were washed three times with D-PBS (200 mu l per well) and permeabilized with 1% Triton X-100 in D-PBS for 15 minutes at room temperature. After three washes in D-PBS, the explants were incubated with FITC-labeled Phalloidin (1:60 from stock, 50 mu l/well) for 45 minutes at room temperature. The plates were covered with aluminum foil as the Phalloidin is light sensitive. After three more washes with D-PBS, the labeled explants were placed in a drop of glycerol on a microscope slide, covered with a glass coverslip and sealed with nail polish. The explants were observed under a Nikon Diaphot-300 inverted fluorescence microscope, using FITC filters and fluorescence optics.

Determination of Hair Cell Number

For each experimental point, 2 to 4 cochlea were used. In each cochlea, the number of hair cells was counted in 2-3 section, 175 mm in length each. Only the sections in the middle turn of the cochlea were analyzed. Each experiment was repeated several times. The numbers of hair cells in control and cisplatin- or neomycin-treated cultures was generated from analyzing 40 cochlea per point.

Results

Hair cells in the floating explant cultures did not die during the experiment period of four days. Thus, the number of phalloidin-stained cells present at the end of the 4 days experiment period, in the absence of ototoxins and treatments, was 105.4 +/- 6.9 (n = 28). Ototoxins added to the explants on the second day post-plating caused significant loss in hair cell number found after 4 days in vitro. Exposure to 35 mu g/ml cisplatin 24 hours after plating caused a loss of about 80 percent of the hair cells: only 20.8% +/- 4.6 (n = 21) of the initial number of hair cells survived (Table 1) and after exposure to 0.8 mM neomycin, only 5.9% +/- 4.7 (n = 23) of the hair cells survived (Table 2). There was a marked difference in the morphology of the organs of Corti between these two treatments: while the treatment with neomycin resulted in almost complete loss of hair cells, those that were spared were still organized in the typical four row structure (3 rows of outer hair cells and one row of inner hair cells). Cisplatin treatment, on the other hand, caused a marked disruption of the four-row-structure and the surviving cells were randomly located.

In cultures that received neurturin at the time of plating (pretreatment), a significant number of hair cells survived the 3-day exposure to ototoxins (from day 2 to day 4). In cultures exposed to cisplatin, treatment with neurturin concentrations as low as 0.1 ng/ml caused an increase in surviving hair cells from 21% (untreated cultures) to 35%. Maximal protective activity was reached with 1 ng/ml neurturin (50% survival) (Table 1). In cultures exposed to neomycin, neurturin at 0.1 ng/ml increased the number of hair cells from 6% to 22%; maximal neurturin activity (22% survival) was seen with 10 ng/ml neurturin (Table 2). Neurturin treatment preserved the four-row morphology in neomycin-treated cultures, but did not prevent its disruption by cisplatin.

TABLE 1

Effect of neurturin on cochlear hair cells exposed to cisplatin

	Hair cell survival (% of untreated)
Cisplatin alone (35 mu g/ml)	20.8 +/- 6.9 n = 28
Cisplatin + Neurturin 0.1 ng/ml	35.5 +/- 9.1 n = 9
Cisplatin + Neurturin 1 ng/ml	50.0 +/- 13.8 n = 5
Cisplatin + Neurturin 10 ng/ml	37.0 +/- 6.1 n = 7
Cisplatin + Neurturin 50 ng/ml	40.8 +/- 5.3 n = 4
Cisplatin + Neurturin 100 ng/ml	46 +/- 10.8 n = 10

Neurturin was introduced to the explant cultures on the day of plating. Cisplatin (35 mu g/ml) was added 24 hours later, and the cultures were incubated for an additional 3 days. The hair cells were stained with FITC-phalloidin. The number of hair cells was counted in the middle turn of the cochlea in 2-3 sections of 175 mu m each. The results are expressed as the percentage of hair cells present in untreated cultures after 4 days in vitro (105.4 +/- 6.9; n = 28). Each number is the mean +/- SD of n cochleas.

TABLE 2

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        Effect of neurturin on cochlear hair cells exposed to neomycin

                                        Hair cell survival
                                        (% of untreated)

        Neomycin alone                   5.9 +/- 4.7 n = 23
        (0.6 mM)

        Neomycin + Neurturin            21.6 +/- 3.1 n = 4
        0.1 ng/ml

        Neomycin + Neurturin            19.0 +/- 3.3 n = 3
        1 ng/ml

        Neomycin + Neurturin            21.6 +/- 5.2 n = 4
        10 ng/ml

        Neomycin + Neurturin            17.4 +/- 3.9 n = 3
        50 ng/ml

        Neomycin + Neurturin            17.0 +/- 1.3 n = 3
        100 ng/ml
```

Neurturin was introduced to the explant cultures on the day of plating. Neomycin (35 mu g/ml) was added 24 hours later, and the cultures were incubated for an additional 3 days. The hair cells were stained with FITC-phalloidin. The number of hair cells was counted in the middle turn of the cochlea in 2-3 sections of 175 mu m each. The results are expressed as the percentage of hair cells present in untreated cultures after 4 days in vitro (105.4 +/- 6.9; n = 28). Each number is the mean +/- SD of n cochleas.

Example 2

Recombinant Production of a Neurturin Protein Product in E. coli

Exemplary neurturin protein products, as depicted in the Figures were expressed in E. coli. Complementary, overlapping oligonucleotides comprising the encoding nucleotide sequence (e.g., FIG. 3) were synthesized such that the codons used were optimized for E. coli expression. The oligonucleotides were annealed and used as templates for PCR procedures as described in PCR Technology, Principles and Applications for DNA Amplification, Henry A. Erlich, ed., Stockton Press, NY, 1989 (Chapter 6, Using PCR to Engineer DNA) the disclosure of which is hereby incorporated by reference. The product of the PCR reaction was the full-length neurturin gene. This DNA fragment was cloned into an expression vector for expression in E. coli. Following DNA sequence verification, the expression plasmid was then transformed into an E. coli host strain.

Example 3

Neurturin Protein Product to Promote Survival of Inner Ear Auditory Neurons (Spiral Ganglion Neurons) and to Protect Against Ototoxins

Materials

The materials to be used in the following Example may be obtained as follows.

Cell Culture Media

High glucose Dulbecco's Modified Eagle's Medium (DMEM; #11965-092), Ham's F12 medium (F 12; #11765-021), B27 medium supplement (#17504-010), penicillin/streptomycin (#15070-014), L-glutamine (#25030-016), Dulbecco's phosphate-buffered saline (D-PBS; #14190-052), mouse laminin (#23017-015), bovine serum albumin and fractionV (#110-18-017) are all from GIBCO/BRL, Grand Island, N.Y. Heat-inactivated horse serum is from HyClone, Logan, Utah. Poly-L-ornithine hydrobromide (P-3655), bovine insulin (I-5500), human transferrin (T-2252), putrescine (P-6024), progesterone (P-6149) and sodium selenite (S-9133) are all from Sigma Chemical Company, Saint-Louis, Mo. Papain, deoxyribonuclease I (DNAase) and ovalbumin (Papain dissociation system) are from Worthington Biochemicals, Freehold, N.J. Falcon sterile 96-well microplates (#3072), tissue culture plastic ware and polypropylene centrifuge tubes are from Beckton-Dickinson, Oxnard, Calif. Nitex 20 mu m nylon mesh (#460) is from Tetko, Elmsford, N.Y. The 4" dissecting forceps and 4" dissecting scissors are from Roboz Surgical, Washington, D.C.

Antibodies and Related Reagents

Neuronal Specific Enolase (NSE) rabbit polyclonal antibody, is from Chemicon (#AB95 1), biotinylated goat anti-rabbit IgG (#BA-1000) and peroxidase-conjugated avidin/biotin complex (ABC Elite; kit PK-6100) are from Vector Laboratories, Burlingame, Calif. 3',3'-diaminobenzidine is from Cappel Laboratories, West Chester, Pa. Superblock blocking buffer in PBS (#37515) is from Pierce, Rockford, Ill. Triton X-100 (X100), Nonidet P-40 (N6507) and hydrogen peroxide (30%, v/v; H1009) are from Sigma. All other reagents are obtained from Sigma Chemical Company (Saint-Louis, Mo.), unless otherwise specified.

Ototoxins

Cisplatin (Platinol-AQ, #NDC 0015-3220-22) is from Bristol-Myers Squibb, Princeton, N.J., sodium salicylate is from J.T. Baker, Phillipsburg, N.J. (#3872-01) and neomycin is from Sigma (#N1 142).

Methods

Preparation of Media

A basal medium is prepared as a 1:1 mixture of DMEM and F12 medium, and is supplemented with B27 medium supplement added as a 50-fold concentrated stock solution. The B27 medium supplement consists of biotin, L-carnitine, corticosterone, ethanolamine, D (+)-galactose, reduced glutathione, linoleic acid, linolenic acid, progesterone, putrescine, retinyl acetate, selenium, T3 (triodo-1-thyronine, DL-alpha-tocopherol; vitamin E), DL-alpha-tocopherol acetate, bovine serum albumin, catalase, insulin, superoxide dismutase and transferrin. L-glutamine is added at a final concentration of about 2 mM, penicillin at about 100 IU/l, and streptomycin at about 100 mg/l. Heat-inactivated horse serum is added to a final concentration of about 2.5 percent, D-glucose is added to a final concentration of about 5 g/l, HEPES buffering agent is added to a final concentration of about 20 mM, bovine insulin is added to a final concentration of about 2.5 mg/ml, and human transferrin is added to a final concentration of about 0.1 mg/ml. After mixing, the pH is adjusted to about 7.3, and the medium is kept at 4o C. The media are prepared fresh just before use in order to minimize inter-experimental variations. Plastic pipettes and containers are used throughout to minimize protein adsorption.

Neurturin Protein Product Solutions

Purified recombinant neurturin protein products (e.g., FIGS. 1 and 3) are prepared as 1 mg/ml solutions in D-PBS (phosphate-buffered saline prepared with distilled water) containing five percent bovine serum albumin. The solutions are stored at - 85o C. in aliquots. Serial dilutions are prepared in 96-well microplates. Ten microliters of ten-fold concentrated neurturin protein product solutions are added to cell cultures containing culture medium (90 mu l). Control cultures received D-PBS with 5 percent albumin (10 mu l). The neurturin protein product treatments are added to the cultures one hour after cells are seeded or 24 hours later, alone or together with the ototoxins.

Ototoxins Preparations

Neomycin is added straight from a stock solution (about 10< - 3 > M) at 10 mu l per well to result in a final concentration of about 10< - 4 > M. Cisplatin is diluted with culture medium from the stock solution (1 mg/ml) to a solution of 20 mu g/ml and added at 10 mu l per well, to result in a final concentration of 2 mu g/ml. Sodium salicylate is prepared from powder to a stock solution of 1M in PBS and further diluted in the culture medium to 100 mM, which results in a 10 mM final concentration when added at 10 mu l/well to the culture.

Culture Substratum

To encourage optimal attachment of spiral ganglion cells on substratum and neurite outgrowth, the microtiter plate surfaces (the culture substratum) are modified by sequential coating with poly-L-omithine followed by laminin in accordance with the following procedure. The plate surfaces are completely covered with a 0.1 mg/ml sterile solution of polyomithine in 0.1 M boric acid (pH 8.4) for at least one hour at room temperature, followed by a sterile wash with Super-Q water. The water wash is then aspirated, and a 10 mu g/ml solution of mouse laminin in PBS is added and incubated at 37o C. for two hours. These procedures are conducted just before using the plates in order to ensure reproducibility of the results.

Preparation of Rat Spiral Ganglion Cell Cultures

Three- to four-week-old Wistar rats (obtained from Jackson Laboratories, Bar Harbor, Ma.) are injected with an overdose of the following solution: ketamine (100 mg/ml); Xylazine (20 mg/ml) and Acopromazine Maleate 910 mg/ml) at 3:3:1 proportions. The rats are then killed by decapitation, and the temporal bone with the cochlea are dissected out and transferred sterilely into PBS with 1.5 g/L glucose on ice. A maximum of cochlea are processed per experiment. The cochlea are opened, and the organ of Corti with the bony modiolus is collected into a 50 ml sterile tube containing 5 ml of dissociation medium (120 units papain and 2000 units DNAase in HBSS). The tissue is incubated for 30 minutes at about 37o C. on a rotary platform shaker at about 200 rpm. The dissociation solution is replaced with a fresh solution, and the incubation is resumed for another 30 min. The cells are then dispersed by trituration through fire-polished Pasteur pipettes, sieved through a 40 mu m Nitex nylon mesh to discard undissociated tissue, and centrifuged for five minutes at 200 x g using an IEC clinical centrifuge. The resulting cell pellet is resuspended in HBSS containing ovalbumin and about 500 units DNAase, layered on top of a four percent ovalbumin solution (in HBSS) and centrifuged for about 6 minutes at 500 x g. The final pellet is resuspended in about 6 ml of the culture medium and seeded at 90 mu l/well in the precoated plates.

Immunohistochemistry of Spiral Ganglion Cells

Spiral ganglion neurons are identified by immunohistochemical staining for neuronal specific enolase (NSE). Cultures of spiral ganglion cells are fixed for about 10 minutes at room temperature with eight percent paraformaldehyde in D-PBS, pH 7.4, added at 100 mu l/well to the culture medium and then replaced by 100 mu l of four percent paraformaldehyde for additional 10 minutes, followed by three washes in D-PBS (200 mu l per 6-mm well). The fixed cultures are then incubated in Superblock blocking buffer in PBS, containing one

percent Nonidet P-40 to increase the penetration of the antibody. The rabbit polyclonal anti-NSE antibodies (Chemicon) are then applied at a dilution of 1:6000 in the same buffer, and the cultures are incubated for two hours at 37o C. on a rotary shaker. After three washes with D-PBS, the spiral ganglion cell-bound antibodies are detected using goat-anti-rabbit biotinylated IgG (Vectastain kit from Vector Laboratories, Burlingame, Calif.) at about a 1:300 dilution. The secondary antibody is incubated with the cells for about one hour at 37o C., and the cells are washed three times with D-PBS. The secondary antibody is then labeled with an avidin-biotin-peroxidase complex diluted at 1:300, and the cells are incubated for about 60 minutes at 37o C. After three more washes with D-PBS, the labeled cell cultures are reacted for 5 minutes in a solution of 0.1 M Tris-HCl, pH 7.4, containing 0.04% 3',3'-diaminobenzidine-(HCl)4, 0.06 percent $NiCl_2$ and 0.02 percent hydrogen peroxide.

Determining Spiral Ganglion Cell Survival

After various times in culture (24 hours, 3 days and 4 days), rat spiral ganglion cell cultures are fixed, processed and immunostained for NSE as described above, and the cultures are then examined with bright-light optics at 200 x magnification. All of the NSE-positive neurons present in a 6-mm well are counted. Viable spiral ganglion cells are characterized as having a round body with a size ranging from 15-40 mu m and bearing neuritic processes. Spiral ganglion cells showing signs of degeneration, such as having irregular, vacuolated perikarya or fragmented neurites, are excluded from the counts (most of the degenerating spiral ganglion cells, however, detached from the culture substratum). Cell numbers are expressed either as cells/6-mm well or as the fold-change relative to control cell density.

Results

Cultures of rat spiral ganglion neurons may be used to demonstrate the effect of neurturin protein product on survival and protection against ototoxins. The spiral ganglion cells are obtained from three to four-week old rat cochlea. The dissociated cells are then seeded into polyornithine-laminin-coated microplates at a density of about 1 cochlea per 2 wells in DMEM/F12 supplemented with B27 medium supplement, 2.5 percent heat-inactivated horse serum, D-glucose, HEPES, insulin and transferrin. The cultures will consist of a mixture of neurons and non-neuronal cells. Preferably, the only neurons present are spiral ganglion neurons, and these may be identified by the presence of NSE immunoreactivity.

The effect of neurturin protein product administration is assessed on the survival and morphological maturation of cultured rat spiral ganglion neurons, as well as on their ability to resist the toxic effect of a known ototoxin such as cisplatin. Cultures of spiral ganglion cells are treated 24 hours after seeding with human recombinant neurturin protein product (ranging from 50 ng/ml to 0.1 ng/ml) alone, or in combination with cisplatin (35 mu g/ml). Twenty four hours after seeding, it is expected that there is no difference in the number of auditory neurons between control cultures and those treated with neurturin at 1 ng/ml and 10 ng/ml. After an additional period of 3 days, treatment with neurturin at a concentration of 1 ng/ml is not expected to result in a significant increase in neuronal cell number. It is envisioned, however, that there will be a marked trophic effect: the neuronal soma are larger and fibers longer and more elaborate than in control cultures. In cultures treated with 10 ng/ml neurturin, about 70% of the neurons present after 24 hours are expected to survive, representing an average 40% increase over control cultures. The trophic effect is expected to be even stronger than in cultures treated with 1 ng/ml neurturin.

Neurturin is also expected to protect spiral ganglion neurons from cisplatin toxicity. Exposure of cultures to 5 mu g/ml cisplatin 24 hours after seeding may result in the loss of about 90% of the initial number (at 24 hours) of the neurons after 4 days in culture. When neurturin is added together with the cisplatin, the number of neurons found after 4 days is expected to be significantly higher. It is also envisioned that this protective effect of neurturin is dose-dependent and that about 60 percent of the neurons that respond to neurturin (about 40% of

the spiral ganglion neuron population) can also be protected against cisplatin toxicity.

Example 4

Neurturin Protein Product to Promote In vivo Survival of Cochlear Hair Cells

The following example describes the inner ear administration of neurturin protein product to protect cochlear hair cells against ototoxicity in an animal model. The neurturin protein product is introduced into the inner ear via a cannula pushed into the scala tympani through a hole drilled in the basal turn of the cochlea. The cannula is connected to an Alzet mini-pump loaded with neurturin protein product (50 ng/ml) at a releasing rate of 0.5 mu l/hour for 14 days. Cisplatin i.m. injections are started two days after the cannulation, at either 1 mg/ml daily for 15 days or at 7.5 mg/kg twice, at a 5 days interval. The experiment is terminated after 27 days. The hair cells are stained with FITC-phalloidin, and their number is determined in the middle turn of the cochlea (in at least 20% of the middle turn part). The results are expressed as the percent of hair cells lost for each individual guinea pig for the neurturin protein product treated ear (right ear) and the untreated ear (left ear).

Materials

The materials to be used in the following Example are obtained as follows

Mini-pump Preparation Materials

Medical vinyl tubing size V/4, catalog No BB317-85, is from Bolab Products ((800) 331-7724). Fisher brand 5 ml plastic pipettes are used. Microlumen Polyimide tubing, catalog #8004853 OG (Tampa, Fla.) is used. Silicone Medical Product MDX 4-4210, is from Dow Corning Corporation, Midland, Mich. Alzet osmotic mini-pump flow moderator and Alzet osmotic mini-pump, Catalog No 2002, are from Alza Corp., Palo Alto, Calif. Tape (TimeMed tape). Prosil-28, Product No 11975-0, is from PCR Incorporated, Gainesville Fla. Purified neurturin protein products are prepared as 50 ng/ml solutions in D-PBS and 0.1% BSA. Sterile 0.1% methylene blue (catalog #M-9140) dissolved in PBS, and mineral oil (catalog #400-5), are from Sigma.

Mini-pump Preparation Procedure

Vinyl tubing is cut into an approximately four inch section and placed in a miniature vise. A piece of the Microlumen Polyimide tube (7 mm) is placed into the end of the vinyl tube. Silicone is mixed by adding approximately 10 parts of base and one part of curing agent. A droplet is placed at the opening of the vinyl tube using a fine probe, and the Microlumen tube is pushed into the vinyl, leaving 3.75 mm length extending from the vinyl tube. Using a drop of Silicone on the probe, a small ball is created around the Microlumen tube, 0.5 mm from the tip, and allowed to dry over night.

The diameter of a 5 ml pipette is increased by applying three concentric layers of tape down the length of the pipette. A constant gap is left where the pipette remains uncovered. V/4 tubing is wrapped around the pipette, and the coils are adjusted so that there are two loose ends of tubing and there is a continuous contact between all coils. Two thin strips of tape are aligned with the edges of the tape on the pipette, to secure the coil in place. Two thin lines of super glue are applied evenly on the coils. After drying for a minimum of one hour, the loose ends are aligned approximately parallel to the pipette and secured in place with one strip of tape. A drop of super glue is applied to secure the tubing to the coils. Following overnight drying, the tape is removed and the coils are slid off the pipette. A flow moderator is inserted into one of the loose ends and secured with one drop of super glue.

The coils are flushed with 1% Prosil-28 in water, rinsed thoroughly with water and then flushed with 70% ethanol. The ethanol is removed by means of a syringe or air vacuum. Coils are left in the desiccator with air vacuum on for at least 30 minutes and are kept overnight in the closed and tight desiccator followed by gas sterilization. During the loading procedure, the coil device is kept horizontal as much as possible to prevent gravity driven movement of the liquids of neurturin protein product, oil, and dye. The formation of air bubbles in the pump or coils is avoided. The pump is submerged in sterile PBS and incubated overnight at 37o C.

The loading of a pump with methylene dye is done by holding the pump in a vertical position. A dye-loaded syringe is inserted completely into the pump, and the dye is injected until the pump overflowed. Injection of any air bubbles into the pump is avoided. A short piece of sterile V/4 tubing is placed onto the Flow Moderator. Neurturin protein product is loaded at a concentration of 50 ng/ml in PBS + 0.1% BSA, in a total volume of 230 mu l, to within about 10 mm of the cannula tip, using a syringe connected with V/4 tubing. For vehicle control experiments, the same volume of PBS + 0.1% BSA is loaded into the pumps. The short piece of V/4 tubing is removed. Mineral oil is then loaded into the coil device with a syringe in such a way that a 2 mm air space and 7 mm of mineral oil are interposed between the pump fluid and the line fluid (infusion fluid). A Flow Moderator is inserted completely into the pump.

Pump Insertion in Inner Ear

Materials

Tissue adhesive glue-Cyanoacrylate, is from Vetbond Tissue Adhesive, 3M Animal Care Products, St. Paul, Minn. Carboxylate cement ESPE Durelon, catalog #03828, is from ESPE-Premier Sales Corp., Norristown Pa. Methyl methacrylate is from Lang Jet Acrylic, Lang Dental MFG, Co., Wheeling, Ill. Dissecting tools are from Roboz Surgical. Xylazine, ketamine and buprenorphine are used. Lubricant Ophthalmic Ointment (AKWA Tears) is from Akorn Inc., Abita Springs La. Xylocaine 2%, catalog No NDC 0186-0160-01, is from ASTRA. Medical Grade Silicone Grease, Art. No. 51.300, is from Unimed. Durelon Pulver powder carboxylate-cement, catalog No. D-82229, is from ESPE, Seefeld. Sulfate ointment (Bacitracin Zinc-neomycin, catalog No. 0168-0012-31) is from Fougera.

Procedure

Albino guinea pigs (250-350 g) are anesthetized with a mixture of xylazine 10 mg/kg, ketamine 40 mg/kg and buprenorphine 0.05 mg/kg. The right ear area is shaved caudally, starting about 2 cm anterior to vertex, 4-5 cm posterior to scapulae and postauricularly. The shaved area is washed with Betadine. Lubricant ophthalmic ointment is applied to both eyes. Xylocaine is injected subcutaneously into the tissue to be incised. Using aseptic technique, a post-auricular incision is made. Using a fine needle, a hole is drilled into the bulla to expose the middle ear cavity and visualize the cochlea. A small hole is drilled manually into the bone wall of the basal turn, below the round window using a fine needle. The tip of the cannula is inserted into the hole until the silicone drop is seated against the bone, which places the cannula tip about midway into the scala tympani canal. A drop of cyanoacrylate is placed at the bulla hole. Carboxylate cement is placed around the cannula over the cyanoacrylate. Once the cement hardens, the placement is confirmed, and the rest of the hole is covered with carboxylate cement on top of a layer of silicone grease. A subcutaneous pocket is made between the scapulae to accommodate the pump which is then inserted. The subcutaneous pocket is rinsed once with 3 ml of a solution of nitrofurazone dissolved in sterile PBS and is then filed with 3 ml of sterile PBS plus 1% Gentamycin to discourage infection. The incision is closed with wound clips after nitrofurazone powder is applied around the wound.

Deafening

Materials

Cisplatin (Platinol-AQ), catalog No NDC 0015-3220-22, is from Bristol-Myers Squibb Laboratories, Princeton. N.J.

Procedure

Injections of cisplatin (i.p.) are started two days after mini-pump implantation. Two paradigms of application are used: either two 7.5 mg/kg injections made at a 5 days interval, or 1 mg/kg daily, for 15 days.

Perfusion

After four weeks, the guinea pigs are deeply anesthetized with a mixture of xylazine and ketamine, and are perfused transcardially with ice-cold PBS followed by ice-cold 4% paraformaldehyde in PBS. Temporal bones are removed, and the bony cochlea is placed in 4% paraformaldehyde for postfixation overnight at 4o C.

Staining

Surface preparation and Phalloidin staining methods are used to stain hair cells. The bony cochlea is opened by a fine needle or #11 blade. Stria vascularis is removed using a fine forceps. In a petri dish filled with PBS, the basal membrane is carefully dissected out from the boney modiolus, using fine needles. Care is taken to remove it intact. The procedure for Phalloidin staining is similar to that performed for the in vitro explants, with the following changes: permeabilization is done for 20-30 minutes, and Phalloidin is added for 90 minutes. Apex, middle turn and basal turn pieces are mounted on a 60 x 22 glass coverslip. A drop of VECTASHIELD mounting medium is added, and the samples are covered with a 22 x 22 mm coverslip and sealed with nail polish to prevent evaporation.

Data Analysis

Each cochlea is examined under microscope with a FITC filter set. Eight segments with the greatest hair cell loss from midturn of basal membrane are selected and photographed using an attached computer printer. Hair cell counts are performed manually, using the photographs. In each animal, hair cell loss in the left ear (as a control, i.e., without neurturin protein product infusion) is compared to hair cell loss in the right ear (neurturin protein product infused).

Results

Cisplatin injections result in a significant loss of hair cells in the cochlea. This loss, in the middle turn sections analyzed in the left ears of three guinea pigs injected with cisplatin at 1 mg/kg daily for 15 days, is expected to be from to 50%. Also in the guinea pig injected with cisplatin at a 7.5 mg/kg twice, instead of the 1 mg/kg daily, there is an anticipated loss of approximately 40% of hair cells in the left ear. The introduction of neurturin into the right inner ear of each of the guinea pigs, is expected to result in a significant reduction in the loss of hair cells. In animals implanted with a mini-pump filed with vehicle instead of neurturin protein product, there is no expected difference in the number of hair cells found in the left ear (untreated ear) and the right ear (implanted) ear.

Example 5

Neurturin Protein Product Injections to Promote In vivo Survival of Cochlear Hair Cells

The following example describes the use of neurturin protein products to protect cochlear hair cells against ototoxicity in an animal model when applied into the middle ear. Neurturin protein product is introduced into the right middle ear by a single injection through the tympanic membrane at a concentration of 1 mg/ml in PBS + 1% BSA in a volume of 125-135 mu l. Cisplatin i.m. injections are started a day after the neurturin protein product injection at 7.5 mg/kg, twice, at a 5 days interval. The experiment is terminated three days after the second cisplatin injection. The hair cells are stained with FITC-phalloidin, and their number determined in the middle turn of the cochlea (in at least 20% of the middle turn part). The results are expressed as the percent of hair cells lost for each individual guinea pig for the neurturin protein product treated ear (right ear) and the untreated (left ear).

Materials

The materials used in this experiment are the same as those used in Example 4.

Procedure

Albino guinea pigs (weighing 600-700 g) are anesthetized with a mixture of xylazine 10 mg/kg, ketamine 40 mg/kg and buprenorphine 0.05 mg/kg. Under a surgical microscope, a hole is made in the tympanic membrane of the right ear by inserting a 27 gauge needle into the membrane. In another location of the tympanic membrane, neurturin protein product (at a concentration of 1 mg/ml in PBS + 1% BSA) is injected into the middle ear cavity so that the whole cavity is full (125-135 mu l). A few animals are injected with vehicle only (PBS + 0.1% BSA) instead of neurturin protein product. The next day, an i.m. injection of cisplatin (7.5 mg/kg) is made. Five days later, a second injection at the same concentration is made. Three days later (8 days of total experiment period), the animals are sacrificed, tissues are fixed and cochlea are analyzed as described in Example 4.

Results

At eight days, the guinea pigs injected with cisplatin are expected to display a significant loss of hair cells in the cochlea. In the left ears, the ears that do not receive neurturin protein product, the loss of hair cells in the middle turn of the cochlea is expected to be 35 to 50%. Injection of neurturin protein product into the cavity of the right middle ear, at 1 mg/ml, is expected to reduce this loss significantly: to about 16 to 30%. Guinea pigs that receive vehicle injections into the right ear instead of neurturin protein product, are not expected to demonstrate a difference in hair cell number between the right (treated) and left (untreated) ear.

Numerous modifications and variations in the practice of the invention are expected to occur to those skilled in the art upon consideration of the foregoing description of the presently preferred embodiments thereof.

- - SEQUENCE LISTING

- - - - (1) GENERAL INFORMATION:

- - (iii) NUMBER OF SEQUENCES: 5

- - - - (2) INFORMATION FOR SEQ ID NO:1:

- - (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 102 amino - -acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

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- - (ii) MOLECULE TYPE: protein
- - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:
- - Ala Arg Leu Gly Ala Arg Pro Cys Gly Leu Ar - -g Glu Leu Glu Val Arg
1 5 - - 10 - - 15
- - Val Ser Glu Leu Gly Leu Gly Tyr Ala Ser As - -p Glu Thr Val Leu Phe
20 - - 25 - - 30
- - Arg Tyr Cys Ala Gly Ala Cys Glu Ala Ala Al - -a Arg Val Tyr Asp Leu
35 - - 40 - - 45
- - Gly Leu Arg Arg Leu Arg Gln Arg Arg Arg Le - -u Arg Arg Glu Arg Val
50 - - 55 - - 60
- - Arg Ala Gln Pro Cys Cys Arg Pro Thr Ala Ty - -r Glu Asp Glu Val Ser
65 - -70 - -75 - -80
- - Phe Leu Asp Ala His Ser Arg Tyr His Thr Va - -l His Glu Leu Ser Ala
85 - - 90 - - 95
- - Arg Glu Cys Ala Cys Val
100
- - - - (2) INFORMATION FOR SEQ ID NO:2:
- - (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 100 amino - -acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
-. - (ii) MOLECULE TYPE: protein
- - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:
- - Pro Gly Ala Arg Pro Cys Gly Leu Arg Glu Le - -u Glu Val Arg Val Ser
1 5 - - 10 - - 15
- - Glu Leu Gly Leu Gly Tyr Thr Ser Asp Glu Th - -r Val Leu Phe Arg Tyr
20 - - 25 - - 30
- - Cys Ala Gly Ala Cys Glu Ala Ala Ile Arg Il - -e Tyr Asp Leu Gly Leu
35 - - 40 - - 45
- - Arg Arg Leu Arg Gln Arg Arg Arg Val Arg Ar - -g Glu Arg Ala Arg Ala
50 - - 55 - - 60
- - His Pro Cys Cys Arg Pro Thr Ala Tyr Glu As - -p Glu Val Ser Phe Leu
65 - -70 - -75 - -80
- - Asp Val His Ser Arg Tyr His Thr Leu Gln Gl - -u Leu Ser Ala Arg Glu
85 - - 90 - - 95
- - Cys Ala Cys Val
100
- - - - (2) INFORMATION FOR SEQ ID NO:3:
- - (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 312 base - -pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
- - (ii) MOLECULE TYPE: DNA (genomic)
- - (ix) FEATURE:
(A) NAME/KEY: CDS
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     (B) LOCATION: 1..309
- -  (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:
- -  ATG GCA CGT CTG GGT GCT CGT - - 45
- -  CTG GGT CTG CGT CGC CTG CGT CAG CGC CGT CG - -C CTG CGT CGC GAA CGT 192
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- -  GTT CGC GCA CAG CCG TGT TGC CGT CCG ACC GC - -A TAC GAA GAC GAA GTT 240
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65 - - 70 - - 75 - - 80
- -  TCC TTC CTG GAC GCT CAC TCC CGT TAC CAC AC - -C GTT CAC GAA CTG TCC 288
Ser Phe Leu Asp Ala His Ser Arg Tyr His Th - -r Val His Glu Leu Ser
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- -  GCA CGT CAC TGT GCG TGT GTT TAA - - - - 312
Ala Arg His Cys Ala Cys Val
100
- - - -  (2) INFORMATION FOR SEQ ID NO:4:
- -  (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 103 amino - -acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear
- -  (ii) MOLECULE TYPE: protein
- -  (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:
- -  Met Ala Arg Leu Gly Ala Arg Pro Cys Gly Le - -u Arg Glu Leu Glu Val
1 5 - - 10 - - 15
- -  Arg Val Ser Glu Leu Gly Leu Gly Tyr Ala Se - -r Asp Glu Thr Val Leu
20 - - 25 - - 30
- -  Phe Arg Tyr Cys Ala Gly Ala Cys Glu Ala Al - -a Ala Arg Val Tyr Asp
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- -  Leu Gly Leu Arg Arg Leu Arg Gln Arg Arg Ar - -g Leu Arg Arg Glu Arg
50 - - 55 - - 60
- -  Val Arg Ala Gln Pro Cys Cys Arg Pro Thr Al - -a Tyr Glu Asp Glu Val
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- -  Ser Phe Leu Asp Ala His Ser Arg Tyr His Th - -r Val His Glu Leu Ser
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- -  Ala Arg His Cys Ala Cys Val
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- - - -  (2) INFORMATION FOR SEQ ID NO:5:
- -  (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 197 amino - -acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
- -  (ii) MOLECULE TYPE: protein
- -  (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:
- -  Met Gln Arg Trp Lys Ala Ala Ala Leu Ala Se - -r Val Leu Cys Ser Ser
1 5 - - 10 - - 15
- -  Val Leu Ser Ile Trp Met Cys Arg Glu Gly Le - -u Leu Leu Ser His Arg
```

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- - Leu Gly Pro Ala Leu Val Pro Leu His Arg Le - -u Pro Arg Thr Leu Asp
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- - Ala Arg Ile Ala Arg Leu Ala Gln Tyr Arg Al - -a Leu Leu Gln Gly Ala
50 - - 55 - - 60
- - Pro Asp Ala Met Glu Leu Arg Glu Leu Thr Pr - -o Trp Ala Gly Arg Pro
65 - -70 - -75 Leu Ar - -g Arg Glu Arg Val Arg
145 1 - -50 1 - -55 1 - -60
- - Ala Gln Pro Cys Cys Arg Pro Thr Ala Tyr Gl - -u Asp Glu Val Ser Phe
165 - - 170 - - 175
- - Leu Asp Ala His Ser Arg Tyr His Thr Val Hi - -s Glu Leu Ser Ala Arg
180 - - 185 - - 190
- - Glu Cys Ala Cys Val
195
```

CLAIMS: What is claimed is:

[*1] 1. A method for treating injury or degeneration of cells of the inner ear comprising administering to a subject a therapeutically effective amount of a neurturin protein product comprising an amino acid sequence set forth in SEQ ID NOs: 1, 2, 3, 4 or 5 wherein said neurturin protein product promotes the survival or function of cochlear hair cells and auditory neurons of the inner ear.

[*2] 2. The method of claim 1, wherein the hearing loss is associated with injury or degeneration of neuroepithelial hair cells in the inner ear.

[*3] 3. The method of claim 1, wherein the hearing loss is associated with injury or degeneration of spiral ganglion neurons.

[*4] 4. The method of claim 1, wherein said auditory neurons are spiral ganglion neurons.

[*5] 5. The method of claim 1, wherein the neurturin protein product has the amino acid sequence set forth in SEQ ID NO:1.

[*6] 6. The method of claim 1, wherein the neurturin protein product has the amino acid sequence set forth in SEQ ID NOs:3 and 4.

[*7] 7. The method of claim 1, wherein the neurturin protein product is (Met< - 1>) neurturin.

[*8] 8. The method of claim 1, wherein the neurturin protein product is administered at a dose of about 1 mu g/kg/day to about 100 mg/kg/day.

[*9] 9. The method of claim 1, wherein the neurturin protein product is administered by cell therapy or gene therapy means wherein cells have been modified to produce and secrete the neurturin protein product.

[*10] 10. The method of claim 8, wherein the cells have been modified ex vivo.

[*11] 11. The method of claim 8, wherein the cells have been modified in vivo.

About LEXIS-NEXIS | Terms and Conditions

Pat. No. 6124322, *

6,124,322

Sep. 26, 2000

Intravenous form of thalidomide for treating immunological diseases

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APPL-NO: 166,953

FILED: Oct. 6, 1998

FOR-PRIOR:
Oct. 6, 1997   Federal Republic of Germany   195 43 968

INT-CL: [7] A01N 43#40; A61K 31#445; C07D 401#00

US-CL: 514#323

CL: 514

SEARCH-FLD: 514#323; 546#200, 201

REF-CITED:

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5,434,170	7/1995	*	Andrulis	514#323

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WO 97/37988	10/1997	*	World Intellectual Property Organization (WIPO)

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Krenn et al. (1993), "Improvements in Solubility and Stability of Thalidomide upon

Complexation with Hydroxypropyl-beta-cyclodextrin". [Abstract]. Chemical Abstracts 118:11605, XP002089567.

PRIM-EXMR: Jones, Dwayne C.

LEGAL-REP: Evenson, McKeown, Edwards & Lenahan, P.L.L.C.

CORE TERMS: thalidomide, enantiomer, parenteral, infusion, glucose, aqueous, pure, diseases, water-soluble, ultrasound, ingredient, concentration, producing, dissolved, isotonic, immunological, administered, endothelium, dissolution, intravenous, solubility, medication, hydrolysis, stomatitis, aphthous, treating, antipode, patient, mixture, aseptic

ABST:
An aqueous thalidomide solution which is suitable as a parenteral form of application of thalidomide, particularly as an intravenous form of application, for treating immunological diseases, and a method of producing the corresponding thalidomide solution.

NO-OF-CLAIMS: 3

EXMPL-CLAIM: 1

NO-OF-FIGURES: 0

NO-DRWNG-PP: 0

SUM:

BACKGROUND OF THE INVENTION

This invention relates to a parenteral form of application of thalidomide and to a method of producing it.

The excessive formation of the cytokinin TNF-a (tumour necrosis factor a) plays a central part in the pathogenesis of graft-versus-host syndrome, aphthous stomatitis, erythema nodosum leprosum, morbus Boeck, morbus Crohn, rheumatoid arthritis and a series of other diseases which are associated with inflammatory symptoms. The basis for the therapy of these diseases consists of the targeted suppression of the release of TNF-a, by administering immunomodulating active ingredients, such as dexamethasone or thalidomide for example. While injectable forms of corticoids such as dexamethasone exist, this has hitherto not been the situation for thalidomide.

In the treatment of aphthous stomatitis, thalidomide has been shown to be superior to classical immunosuppressants. Examples of diseases in which thalidomide has exhibited good efficacy without resulting in a general immunosuppression include cutaneous lupus erythematosus, pyoderma gangrenosum and orogenital ulcers with morbus Behcet, as well as ulcerations in HIV-infected patients, which do not differ histologically from aphthous ulcers and in which-in contrast to the majority of HIV-associated mucocutaneous lesions-no microbial instigators can be detected. As distinct from stomatitis aphthosa, these lesions, which can be characterized as major aphthae, can occur in the entire digestive tract, and when located in the pharyngeal space or the esophagus make the absorption of food difficult, and also make the taking of oral medication difficult, due to the pain which they cause. The pathogenetic factors are endogenous mediators which have effects on the endothelium and on circulating leukocytes. Under the influence of locally-formed TNF-a and other cytokinins, there is a marked increase in the adhesiveness of the endothelium in relation to leukocytes, which makes a definitive contribution to the development of venous vasculitis. Substances which, like thalidomide, suppress this alteration of the endothelium without at the same time

blocking the specific cellular immune defense, can constitute an important advance in therapy.

In severe cases of pharyngeal or esophageal ulcers, in which the taking of oral medication is made difficult, or in which this may even be impossible, and in cases of HIV-associated pathology in which severe symptoms of diarrhoea make the use of oral medication unpredictable, it is appropriate to administer active ingredients parentally. However, the low solubility of thalidomide in water (0.012 mg/ml; Arch. Pharm. 321, 371 (1988)) constitutes an obstacle to the parenteral administration of this active ingredient. There has, therefore, been no lack of attempts to develop water-soluble forms of administration.

Water-soluble thalidomide derivatives are known from DE 4,211,812. These thalidomide derivatives have a considerably higher solubility in water than that of thalidomide and are suitable for parenteral administration.

In addition, thalidomide prodrugs have been proposed for parenteral application which can be administered in water-soluble form in the physiological pH range and which are toxicologically harmless (DE 19,613,976). A disadvantage here is that both types of the aforementioned compounds involve higher production costs than the costs for the production of thalidomide.

SUMMARY OF THE INVENTION

The underlying object of the present invention consisted of developing a water-soluble form of application of thalidomide.

Another object was to provide a form of application which would be stable in a form dissolved in water.

Yet another object was to provide a water-soluble form of thalidomide having non-physiological physicochemical properties which would not give rise to toxicological effects.

It has been found that under certain conditions the requirements imposed on the form of application to be developed can be fulfilled by the use of pure enantiomers of thalidomide. As used herein the term "pure enantiomer" refers to a -orm which contains less than 1% of its optical antipode.

The enantiomers of thalidomide have a solubility in water which is higher by a factor of 6 than that of the racemate. The production of aqueous solutions is not practicable, however, due to the tendency of thalidomide to undergo spontaneous hydrolysis. However, if the pH of aqueous solutions falls within a pH range which is less than or equal to 5.5, hydrolysis does not occur.

According to the current state of knowledge, it is not possible to associate a defined isomer with the mode of action of thalidomide against immunological diseases. Pure enantiomers of thalidomide are converted back into the racemate in vitro and in vivo. Therefore, the antipode is also formed immediately after the parenteral administration of one of the isomers of thalidomide in vivo. An equilibrium is established after about 4 hours.

The present invention accordingly relates to a solution, which is suitable for parenteral application, of one of the two thalidomide enantiomers [See Original Patent for Chemical Structure Diagram]

wherein this solution is an aqueous solution with a pH less than or equal to 5.5 and contains glucose as a constituent. According to the invention, one of the two thalidomide enantiomers

is dissolved in isotonic glucose solution. The definition of the invention comprises both solutions of (+)-(R)-thalidomide and solutions of (-)-(S)-thalidomide, which can be used individually or alternatively for parenteral application, particularly for intravenous administration.

Suitable injectable forms of application of thalidomide are those which have a content of active ingredient of at least 0.2 mg/ml.

The present invention further relates to a method of producing the aqueous thalidomide solution. According to this aspect of the invention, (+)-(R)-thalidomide or (-)-(S)-thalidomide is added in pure form to an isotonic glucose solution with a pH of 4 to 5, and this mixture is shaken until complete dissolution of the respective thalidomide enantiomer has occurred, is subsequently treated with ultrasound and is filtered under aseptic conditions.

The form of application according to the invention is toxicologically harmless for both rapid and slow infusion (10 ml/min).

Pharmaceutical compositions according to the invention comprise glucose in addition to one of the enantiomers of thalidomide. Other adjuvant substances may optionally be added to the thalidomide solution. The choice of these further adjuvant substances and the amounts to be used depend on exactly how the pharmaceutical composition is to be administered.

The amount of active ingredient to be administered to the patient, which depends on the weight of the patient, on the type of parenteral administration, on the indication and on the degree of severity of the illness, is usually between 0.1 and 1 mg/kg.

DETDESC:

EXAMPLES

Example 1

In order to produce an infusion solution in a concentration of 200 mu g/ml, 70 mg (+)-(R)-thalidomide in 350 ml of a 5% glucose solution for infusions (pH 4 to 5) were introduced into a glass infusion bottle. The mixture was thoroughly shaken and treated for 15 minutes with ultrasound. Since the dissolved thalidomide concentration depends on the intensity of shaking and of the ultrasound treatment, both steps were repeated until complete dissolution was achieved. The water temperature in the ultrasonic bath reached a maximum of 33o C. The solution was filtered under aseptic conditions through a Millex GS sterile filter with a pore size of 0.22 mu m (Millipore S. A., Molsheim, France) into a sterile glass infusion bottle. The solution was stored at room temperature. The pH of the final solution was 5.5.

The duration of the ultrasound treatment can be reduced by using a solution of the pure enantiomer in ethanol. This has an initial concentration which is higher by a factor of 5 to 10.

Example 2

In order to produce an infusion solution in a concentration of 200 mu g/ml, 70 mg (-)-(S)-thalidomide in 350 ml glucose solution for infusions (pH 4 to 5) were introduced into a glass infusion bottle. The procedure employed was as in Example 1. The pH of the final solution was 5.5.

Stability Testing

Portions for analysis were removed daily from the solutions on 10 successive days. After 10 days, the respective thalidomide enantiomers were still completely intact, without hydrolysis having occurred. After this period of time, the thalidomide enantiomers contained less than 1% of their optical antipodes.

The foregoing description and examples have been set forth merely to illustrate the invention and are not intended to be limiting. Since modifications of the disclosed embodiments incorporating the spirit and substance of the invention may occur to persons skilled in the art, the invention should be construed broadly to include all variations falling within the scope of the appended claims and equivalents thereof.

CLAIMS: What is claimed is:

[*1] 1. An aqueous thalidomide solution comprising a pure enantiomer of thalidomide selected from the group consisting of (+)-(R)-thalidomide and (-)-(S)-thalidomide, dissolved in an isotonic glucose solution, said thalidomide solution having a pH of at most 5.5.

[*2] 2. An aqueous thalidomide solution according to claim 1, wherein said thalidomide solution contains at least 0.2 mg/ml of said pure enantiomer of thalidomide.

[*3] 3. A method of producing an aqueous thalidomide solution, said method comprising the steps of:

adding a pure enantiomer of (+)-(R)-thalidomide or (-)-(S)-thalidomide to an isotonic glucose solution with a pH of 4 to 5;

shaking the resulting mixture until complete dissolution of the respective thalidomide enantiomer has occurred;

treating the thalidomide solution with ultrasound, and

filtering the thalidomide solution under aseptic conditions.

Source: All Sources : / . . . / : **Utility, Design and Plant Patents** ⓘ
Terms: **patno is (5,837,681)** (Edit Search)

*Pat. No. 5837681, ***

5,837,681

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Nov. 17, 1998

Method for treating sensorineural hearing loss using glial cell line-derived neurotrophic factor (GDNF) protein product

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CORE TERMS: protein, cell, neuron, variant, hair, polymer, residue, sequence, amino acid, inner ear, spiral, ganglion, auditory, molecule, cisplatin, hearing loss, cochlea, neurotrophic, medium, administered, sensorineural, preparation, organ, gene, site, polyethylene glycol, membrane, reactive, explant, substitution

ABST:
The present invention relates generally to methods for preventing and/or treating injury or degeneration of cochlear hair cells and spiral ganglion neurons by administering glial cell line-derived neurotrophic factor (GDNF). The invention relates more specifically to methods for treating sensorineural hearing loss.

NO-OF-CLAIMS: 8

EXMPL-CLAIM: 1

NO-OF-FIGURES: 1

NO-DRWNG-PP: 1

SUM:

BACKGROUND OF THE INVENTION

The present invention relates generally to methods for preventing and/or treating injury or degeneration of inner ear sensory cells, such as hair cells and auditory neurons, by administering glial cell line-derived neurotrophic factor (GDNF) protein product. The invention

relates specifically to methods for preventing and/or treating hearing loss due to variety of causes.

Neurotrophic factors are natural proteins, found in the nervous system or in non-nerve tissues innervated by the nervous system, that function to promote the survival and maintain the phenotypic differentiation of certain nerve and/or glial cell populations (Varon et al., Ann. Rev. Neuroscience, 1:327, 1979; Thoenen et al., Science, 229:238, 1985). Because of this physiological role, neurotrophic factors are useful in treating the degeneration of such nerve cells and the loss of differentiated function that results from nerve damage. Nerve damage is caused by conditions that compromise the survival and/or proper function of one or more types of nerve cells, including: (1) physical injury, which causes the degeneration of the axonal processes (which in turn causes nerve cell death) and/or nerve cell bodies near the site of injury, (2) temporary or permanent cessation of blood flow (ischemia) to parts of the nervous system, as in stroke, (3) intentional or accidental exposure to neurotoxins, such as the cancer and AIDS chemotherapeutic agents cisplatinum and dideoxycytidine, respectively, (4) chronic metabolic diseases, such as diabetes or renal dysfunction, or (5) neurodegenerative diseases such as Parkinson's disease, Alzheimer's disease, and Amyotrophic Lateral Sclerosis, which result from the degeneration of specific neuronal populations. In order for a particular neurotrophic factor to be potentially useful in treating nerve damage, the class or classes of damaged nerve cells must be responsive to the factor. It has been established that all neuron populations are not responsive to or equally affected by all neurotrophic factors.

The first neurotrophic factor to be identified was nerve growth factor (NGF). NGF is the first member of a defined family of trophic factors, called the neurotrophins, that currently includes brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), NT-4/5, and NT-6 (Thoenen, Trends. Neurosci., 14:165-170, 1991; Snider, Cell, 77:627-638, 1994; Bothwell, Ann. Rev. Neurosci., 18:223-253, 1995). These neurotrophins are known to act via the family of trk tyrosine kinase receptors, i.e., trkA, trkB, trkC, and the low affinity p75 receptor (Snider, Cell, 77:627 -638, 1994; Bothwell, Ann. Rev. Neurosci., 18:223-253, 1995; Chao et al., TINS 18:321-326, 1995).

Glial cell line-derived neurotrophic factor (GDNF) is a recently discovered protein identified and purified using assays based upon its efficacy in promoting the survival and stimulating the transmitter phenotype of mesencephalic dopaminergic neurons in vitro (Lin et al., Science, 260:1130-1132, 1993). GDNF is a glycosylated disulfide-bonded homodimer that has some structural homology to the transforming growth factor-beta (TGF- beta) super family of proteins (Lin et al., Science, 260:1130-1132, 1993; Krieglstein et al., EMBO J., 14:736-742, 1995; Poulsen et al., Neuron, 13:1245-1252, 1994). GDNF mRNA has been detected in muscle and Schwann cells in the peripheral nervous system (Henderson et al., Science, 266:1062-1064, 1994; Trupp et al., J. Cell Biol., 130:137-148, 1995) and in type I astrocytes in the central nervous system (Schaar et al., Exp. Neurol., 124:368-371, 1993). In vivo, treatment with exogenous GDNF stimulates the dopaminergic phenotype of substantia nigra neurons and restores functional deficits induced by axotomy or dopaminergic neurotoxins in animal models of Parkinson's disease (Hudson et al., Brain Res. Bull., 36:425-432, 1995; Beck et al., Nature, 373:339-341, 1995; Tomac et al., Nature, 373:335-339, 1995; Hoffer et al., Neurosci. Lett., 182:107-111, 1994). Although originally thought to be relatively specific for dopaminergic neurons, at least in vitro, evidence is beginning to emerge indicating that GDNF may have a larger spectrum of neurotrophic targets besides mesencephalic dopaminergic and somatic motor neurons (Yan and Matheson, Nature 373:341-344, 1995; Oppenheim et al., Nature, 373:344-346, 1995; Matheson et al., Soc. Neurosci. Abstr, 21, 544, 1995; Trupp et al., J. Cell Biol., 130:137-148, 1995). In particular, GDNF was found to have neurotrophic efficacy on brainstem and spinal cord cholinergic motor neurons, both in vivo and in vitro (Oppenheim et al., Nature, 373:344-346, 1995; Zurn et al., Neuroreport, 6:113-118, 1994; Yan et al., Nature, 373: 341-344, 1995; Henderson et al., Science, 266:1062-1064, 1994), on retinal neurons, such as

photoreceptors in vitro (currently pending U.S. application Ser. No. 08/564,833 by Louis, filed Nov. 29, 1995) and retinal ganglion cells both in vitro and in vivo (currently pending U.S. application Ser. No. 08/564,458 by Yan, filed Nov. 29, 1995 and both in vitro and in vivo on sensory neurons from the dorsal root ganglion both (currently pending U.S. application Ser. No. 08/564,844 (by Yan et al.) filed Nov. 29, 1995).

Of general interest to the present invention is WO93/06116 (Lin et al., Syntex-Synergen Neuroscience Joint Venture), published Apr. 1, 1993, which reports that GDNF is useful for the treatment of nerve injury, including injury associated with Parkinson's disease. Also of interest are a report in Schmidt-Kastner et al., Mol. Brain Res., 26:325-330, 1994 that GDNF mRNA became detectable and was upregulated after pilocarpine-induced seizures; reports in Schaar et al., Exp. Neurol., 124:368-371, 1993 and Schaar et al., Exp. Neurol., 130:387-393, 1994 that basal forebrain astrocytes expressed moderate levels of GDNF mRNA under culture conditions, but that GDNF did not alter basal forebrain ChAT activity; and a report in currently pending U.S. application Ser. No. 08/535,682 filed Sep. 28, 1995 that GDNF is useful for treating injury or degeneration of basal forebrain cholinergic neurons. GDNF has not previously been shown to promote survival, regeneration or protection against degeneration of inner ear cells such as hair cells and auditory neurons.

The neuroepithelial hair cells in the organ of Corti of the inner ear, transduce sound into neural activity, which is transmitted along the cochlear division of the eighth cranial nerve. This nerve consists of fibers from three types of neurons (Spoendlin, H. H. In: Friedmann, I. Ballantyne, J., eds. Ultrastructural Atlas of the Inner Ear; London, Butterworth, pp. 133-164, 1984): 1) afferent neurons, which lie in the spiral ganglion and connect the cochlea to the brainstem. 2) efferent olivocochlear neurons, which originate in the superior olivary complex and 3) autonomic adrenergic neurons, which originate in the cervical sympathetic trunk and innervate the cochlea. In the human, there are approximately 30,000 afferent cochlear neurons, with myelinated axons, each consisting of about 50 lamellae, and 4-6 mu m in diameter. This histologic structure forms the basis of uniform conduction velocity, which is an important functional feature. Throughout the length of the auditory nerve, there is a trophic arrangement of afferent fibers, with 'basal' fibers wrapped over the centrally placed 'apical' fibers in a twisted rope-like fashion. Spoendlin (Spoendlin, H. H. In: Naunton, R. F., Fernadex, C. eds. Evoked Electrical Activity in the Auditory Nervous System. London, Academic Press, pp. 21-39, 1978) identified two types of afferent neurons in the spiral ganglion on the basis of morphologic differences: type I cells (95%) are bipolar and have myelinated cell bodies and axons that project to the inner hair cells. Type II cells (5%) are monopolar with unmyelinated axons and project to the outer hair cells of the organ of Corti. Each inner hair cell is innervated by about 20 fibers, each of which synapses on only one cell. In contrast, each outer hair cell is innervated by approximately six fibers, and each fiber branches to supply approximately 10 cells. Within the cochlea, the fibers divide into: 1) an inner spiral group, which arises primarily ipsilaterally and synapses with the afferent neurons to the inner hair cells, and 2) a more numerous outer radial group, which arises mainly contralaterally and synapses directly with outer hair cells. There is a minimal threshold at one frequency, the characteristic or best frequency, but the threshold rises sharply for frequencies above and below this level (Pickles, J. O. In: Introduction to the Physiology of Hearing. London, Academic Press, pp. 71-106, 1982). Single auditory nerve fibers therefore appear to behave as band-pass filters. The basilar membrane vibrates preferentially to different frequencies, at different distances along its length, and the frequency selectivity of each cochlear nerve fiber is similar to that of the inner hair cell to which the fiber is connected. Thus, each cochlear nerve fiber exhibits a turning curve covering a different range of frequencies from its neighboring fiber (Evans, E. F. In: Beagley H. A. ed. Auditory investigation: The Scientific and Technological basis. New York, Oxford University Press, 1979). By this mechanism, complex sounds are broken down into component frequencies (frequency resolution) by the filters of the inner ear.

Hearing loss of a degree sufficient to interfere with social and job-related communications is

among the most common chronic neural impairments in the US population. On the basis of health-interview data (Vital and health statistics. Series 10. No. 176. Washington, D.C. (DHHS publication no. (PHS) 90-1504), it is estimated that approximately 4 percent of people under 45 years of age and about 29 percent of those 65 years or over have a handicapping loss of hearing. It has been estimated that more than 28 million Americans have hearing impairment and that as many as 2 million of this group are profoundly deaf (A report of the task force on the National Strategic plan. Bethesda, Md.: National Institute of Health, 1989). The prevalence of hearing loss increases dramatically with age. Approximately 1 per 1000 infants has a hearing loss sufficiently severe to prevent the unaided development of spoken language (Gentile, A. et al. Characteristics of persons with impaired hearing: United States, 1962-1963. Series 10. No. 35. Washington, D.C.: Government printing office, 1967 (DHHS publication no. (PHS) 1000) (Human communication and its disorders: an overview. Bethesda, Md.: National Institutes of health, 1970). More than 360 per 1000 persons over the age of 75 have a handicapping hearing loss (Vital and health statistics. Series 10. No. 176. Washington, D.C. (DHHS publication no. (PHS) 90-1504).

It has been estimated that the cost of lost productivity, special education, and medical treatment may exceed $ 30 billion per year for disorders of hearing, speech and language (1990 annual report of the National Deafness and other Communication Disorders Advisory Board. Washington, D.C.: Government Printing Office, 1991. (DHHS publication no. (NIH) 91-3189). The major common causes of profound deafness in childhood are genetic disorders and meningitis, constituting approximately 13 percent and 9 percent of the total, respectively (Hotchkiss, D. Demographic aspects of hearing impairment: questions and answers. 2nd ed. Washington, D.C.: Gallaudet University Press, 1989). In approximately 50 percent of the cases of childhood deafness, the cause is unknown, but is likely due to genetic causes or predisposition(Nance W E, Sweeney A. Otolaryngol. Clin. North Am 1975; 8: 19-48).

Impairment anywhere along the auditory pathway, from the external auditory canal to the central nervous system, may result in hearing loss. The auditory apparatus can be subdivided into the external and middle ear, inner ear and auditory nerve and central auditory pathways. Auditory information in humans is transduced from a mechanical signal to a neurally conducted electrical impulse by the action of approximately 15,000 neuroepithelial cells (hair cells) and 30,000 first-order neurons (spiral ganglion cells) in the inner ear. All central fibers of spiral ganglion neurons form synapses in the cochlear nucleus of the pontine brainstem. The number of neurons involved in hearing increases dramatically from the cochlea to the auditory brain stem and the auditory cortex. All auditory information is transduced by only 15,000 hair cells, of which the so-called inner hair cells, numbering 3500, are critically important, since they form synapses with approximately 90 percent of the 30,000 primary auditory neurons. Thus, damage to a relatively few cells in the auditory periphery can lead to substantial hearing loss. Hence, most causes of sensorineural loss can be ascribed to lesions in the inner ear (Nadol, J. B., New England Journal of Medicine, 1993, 329: 1092-1102).

Hearing loss can be on the level of conductivity, sensorineural and central level. Conductive hearing loss is caused by lesions involving the external or middle ear, resulting in the destruction of the normal pathway of airborne sound amplified by the tympanic membrane and the ossicles to the inner ear fluids. Sensorineural hearing loss is caused by lesions of the cochlea or the auditory division of the eight cranial nerve. Central hearing loss is due to lesions of the central auditory pathways. These consist of the cochlear and dorsal olivary nucleus complex, inferior colliculi, medial geniculate bodies, auditory cortex in the temporal lobes and interconnecting afferent and efferent fiber tracts (Adams R. D. and Maurice, V. Eds. in: Principles of Neurology. 1989. McGraw-Hill Information services Company. PP 226-246).

As mentioned previously, at least 50 percent of cases of profound deafness in childhood have genetic causes (Brown, K. S. Med. Clin. North AM. 1969; 53: 741-72). If one takes into consideration the probability that genetic predisposition is a major causative factor in

presbycusis-or age-related hearing loss-which affects one third of the population over 75 years of age (Nadol, J. B. In: Beasley D S, Davis G A, eds. Aging: Communication Processes and Disorders. New York: Grune & Stratton, 1981:63-85), genetic and hereditary factors are probably the single most common cause of hearing loss. Genetic anomalies are much more commonly expressed as sensorineural hearing loss than as conductive hearing loss. Genetically determined sensorineural hearing loss is clearly a major, if not the main cause of sensorineural loss, particularly in children (Nance W E, Sweeney A. Otolaryngol. Clin. North Am 1975; 8: 19-48). Among the most common syndromal forms of sensorineural loss are Waardenburg's syndrome, Alport's syndrome and Usher's syndrome.

A variety of commonly used drugs have ototoxic properties. The best known are the aminoglycoside antibiotics (Lerner, S. A. et al eds. Aminoglycoside ototoxicity. Boston: Little, Brown, 1981; Smith, C. R. et al. N Engl. J. Med. 1980; 302: 1106-9), loop diuretics (Bosher, S. K., Acta Otolaryngol. (Stockh) 1980; 90: 4-54), salicylates (Myers, E. N. at al. N Engl. J. Med. 1965; 273:587-90) and antineoplastic agents such as cisplatin (Strauss, M. at al. Laryngoscope 1983; 143:1263.5). Ototoxicity has also been described during oral or parenteral administration of erythromycin (Kroboth, P. D. at al. Arch. Intern Med. 1983; 1:169-79; Achweitzer, V. G., Olson, N. Arch. Otolaryngol. 1984; 110:258-60).

Most ototoxic substances cause hearing loss by damaging the cochlea, particularly the auditory hair cells and the stria vascularis, a specialized epithelial organ within the inner ear, that is responsible for the homeostasis of fluids and electrolytes (Nadol, J. B. New England J. Med. 1993, 329: 1092-1102). Secondary neural degeneration may occur many years after an ototoxic event affecting the hair cells. There is evidence that some ototoxic substances may be selectively concentrated within the inner ear, resulting in progressive sensorineural loss despite the discontinuation of systemic administration (Federspil, P. at al. J. Infect. Dis. 1976; 134 Suppl: S200-S205)

Trauma due to acoustic overstimulation is another leading cause of deafness. There is individual susceptibility to trauma from noise. Clinically important sensorineural hearing loss may occur in some people exposed to high-intensity noise, even below levels approved by the Occupational Safety and Health Agency (Osguthorpe, J. D. ed. Washington D.C.: American Academy of Otolaryngology-Head and Neck Surgery Foundation, 1988).

Demyelinating processes, such as multiple sclerosis, may cause sensorineural hearing loss (Noffsinger, D at al. Acta Otolaryngol Suppl (Stockh) 1972; 303:1-63). More recently, a form of immune-mediated sensorineural hearing loss has been recognized (McCabe, B. F. Ann Otol Rhinol Laryngol 1979; 88:585-9). The hearing loss is usually bilateral, is rapidly progressive (measured in weeks and months), and may or may not be associated with vestibular symptoms.

A variety of tumors, both primary and metastatic, can produce either a conductive hearing loss, or a sensorineural hearing loss, by invading the inner ear or auditory nerve (Houck, J. R. et al. Otolaryngol Head Neck Surg 1992; 106:92-7). A variety of degenerative disorders of unknown cause can produce sensorineural hearing loss. Meniere's syndrome (Nadol, J. B. ed. Meniere's disease: pathogenesis, pathophysiology, diagnosis, and treatment. Amsterdam: Kugler & Ghedini 1989), characterized by fluctuating sensorineural hearing loss, episodic vertigo, and tinnitus, appears to be caused by a disorder of fluid homeostasis within the inner ear, although the pathogenesis remains unknown. Sudden idiopathic sensorineural hearing loss (Wilson, W. R. at al. Arch Otolaryngol 1980; 106:772-6), causing moderate-to-severe sensorineural deafness, may be due to various causes, including inner ear ischemia and viral labyrinthitis.

Presbycusis, the hearing loss associated with aging, affects more than one third of persons over the age of 75 years. The most common histopathological correlate of presbycusis is the loss of neuroepithelial (hair) cells, neurons, and the stria vascularis of the peripheral auditory

system (Schuknecht H. F. Pathology of the Ear. Cambridge, Mass: Harvard University Press, 1974:388-403). Presbycusis is best understood as resulting from the cumulative effects of several noxious influences during life, including noise trauma, ototoxicity and genetically influenced degeneration.

Certain neurotrophic factors have been shown to regulate neuronal differentiation and survival during development (Korsching S. J. Neurosci. 13:2739-2748, 1993) and to protect neurons from injury and toxins in adult (Hefti, Neurosci. 6:2155-2162, 1986; Apfel et al., Ann Neurol 29:87-89, 1991; Hyman et al., Nature 350:230-233, 1991; Knusel et al., J. Neurosci. 12:4391-4402, 1992; Yan et al., Nature, 360:753-755, 1992; Koliatsos et al., Neuron, 10:359-367, 1993). In situ hybridization studies indicate that mRNAs for the neurotrophin receptors TrkB and TrkC are expressed by developing cochleovestibular ganglia (Ylikoski et al., Hear. Res. 65:69-78 1993; Schecterson et al., Hearing Res. 73: 92-100 1994) and that mRNAs for BDNF and NT-3 are found in the inner ear, including the organ of Corti (Pirvola et al., Proc. Natl. Acad. Sci. USA 89: 9915-9919, 1992; Schecterson et al., Hearing Res. 73: 92-100 1994; Wheeler et al., Hearing Res. 73: 46-56, 1994). The physiological role of BDNF and NT-3 in the development of the vestibular and auditory systems was investigated in mice that carry a deleted BDNF and/or NT-3 gene (Ernfors et al., Neuron 14: 1153-1164 1995). In the cochlea, BDNF mutants lost type-2 spiral neurons, causing an absence of outer hair cell innervation. NT-3 mutants showed a paucity of afferents and lost 87 percent of spiral neurons, presumably corresponding to type-1 neurons, which innervate inner hair cells.

Double mutants had an additive loss, lacking all vestibular and spiral neurons. The requirement of TrkB and TrkC receptors for the survival of specific neuronal populations and the maintenance of target innervation in the peripheral sensory system of the inner ear was demonstrated by studying mice carrying a germline mutation in the tyrosine kinase catalytic domain of these genes (Schimmang et al., Development, 121: 3381-3391 1995). Gao et al., (J. Neurosci. 15: 5079-5087, 1995) showed survival-promoting potency of NT-4/5, BDNF and NT-3 for rat postnatal spiral ganglion neurons in dissociated cultures and that NT-4/5 protected these neurons from neurotoxic effects of the anti-cancer drug, cisplatin. Also, BDNF and NT-3 have been shown to support the survival of adult rat auditory neurons in dissociated cultures (Lefebvre et al., NeuroReport 5: 865-868, 1994).

There have been no previous reports of the use of GDNF in the treatment of hearing loss. Since hearing impairment is a serious affliction, the identification of any agent and treatment method that can protect the auditory neurons and hair calls from damage would be of great benefit.

SUMMARY OF THE INVENTION

The present invention provides methods for treating sensorineural hearing loss comprising administering to a subject having a lesion in the inner ear a therapeutically effective amount of a glial cell line-derived neurotrophic factor (GDNF) protein product. For example, the hearing loss may be associated with injury or degeneration of neuroepithelial hair cells (cochlear hair cells) or spiral ganglion neurons in the inner ear.

The present invention is based on the discoveries that hair cells respond to GDNF by resisting the toxic effects of ototoxins, such as cisplatin and neomycin, and that auditory neurons also respond to GDNF by resisting the toxic effects of variety of ototoxins, such as for example cisplatin, neomycin, and sodium salicylate. Thus, a therapeutically effective amount GDNF protein product may be administered to promote the protection, survival or regeneration of hair cells and spiral ganglion neurons.

It has also been discovered that lesions or disturbances to the vestibular apparatus may also

be treated by administering to a subject having such a lesion or disturbance a therapeutically effective amount of a GDNF protein product. Such lesions may result in dizziness, vertigo or loss of balance.

It is contemplated that such GDNF protein products would include a GDNF protein such as that depicted by the amino acid sequence set forth in FIG. 1 (SEQ ID NO:1), as well as variants and derivatives thereof. It is also contemplated that such GDNF protein products would include [Met< - 1>]GDNF.

According to the invention, the GDNF protein product may be administered parenterally at a dose ranging from about 1 mu g/kg/day to about 100 mg/kg/day, typically at a dose of about 0.1 mg/kg/day to about 25 mg/kg/day, and usually at a dose of about 5 mg/kg/day to about 20 mg/kg/day. It is also contemplated that, depending on the individual patient's needs and route of administration, the GDNF protein product may be given at a lower frequency such as weekly or several times per week, rather than daily. It is further contemplated that GDNF protein product may be administered directly into the middle ear or the inner ear. One skilled in the art will appreciate that with such administration of a smaller amount of GDNF protein product may be used, for example, a direct middle ear or inner-ear administration dose in the range of about 1 mu g/ear to about 1 mg/ear in a single injection or in multiple injections.

It is further contemplated that GDNF protein product be administered in combination or conjunction with an effective amount of a second therapeutic agents, such as BDNF and NT-3. The invention also provides for the use of GDNF protein product in the manufacture of a medicament or pharmaceutical composition for the treatment of injury or degeneration of hair cells and auditory neurons for the variety of causes of sensorineural hearing loss. Such pharmaceutical compositions include topical, oral or middle and inner ear GDNF protein product formulations or in combination with cochlear implants.

It will also be appreciated by those skilled in the art that the administration process can be accomplished via cell therapy and gene therapy means, as further described below. For example, in a gene therapy means cells have been modified to produce and secrete the GDNF protein product. The cells may be modified vivo or in vivo. Numerous additional aspects and advantages of the invention will become apparent to those skilled in the art upon consideration of the following detailed description of the invention which describes presently preferred embodiments thereof.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a method for preventing and/or treating sensorineural hearing loss by administering a therapeutically effective amount of glial cell line-derived neurotrophic factor (GDNF) protein product. According to one aspect of the invention, methods are provided for treating damaged hair cells and auditory neurons by administering a therapeutically effective amount of GDNF protein product by means of a pharmaceutical. composition, the implantation of GDNF-expressing cells, or GDNF gene therapy. The invention may be practiced using any biologically active GDNF protein product, including a GDNF protein represented by the amino acid sequence set forth in SEQ ID NO:1, including variants and derivatives thereof. In addition to oral, parenteral or topical delivery of the GDNF protein product, administration via cell therapy and gene therapy procedures is contemplated.

The present invention is based on the initial discoveries that GDNF protects hair cells from ototoxins-induced cell death in explant cultures of rat's cochlea and dissociated spiral ganglion neurons from adult rat in culture. It is contemplated that administration of exogenous GDNF protein product will protect hair cells and spiral ganglion neurons from

traumatic damage (such as noise trauma and acute or chronic treatments of cisplatin and aminoglycoside antibiotics) or from damage resulting from a lack of neurotrophic factors caused by interruption of transport of the factors from the axon to the cell body. Such treatment is expected to allow hair cells and/or auditory neurons to tolerate intermittent insults from either environmental noise trauma, treatments with ototoxins and to slow down the progressive degeneration of the auditory neurons and hair cells, that is responsible for hearing loss in pathological conditions such as presbycusis (age-related hearing loss), inherited sensorineural degeneration, and post-idiopathic hearing losses and to preserve the functional integrity of the inner ear. It will also support the auditory neurons for a better and longer performance of cochlear implants.

According to the invention, the GDNF protein product may be administered into the middle ear at a dose ranging from about 1 mu g/kg/day to about 100 mg/kg/day, typically at a dose of about 0.1 mg/kg/day to about 25 mg/kg/day, and usually at a dose of about 5 mg/kg/day to about 20 mg/kg/day. GDNF protein product may be administered directly into the inner ear in cases where invasion of the inner ear is already in place such as in the procedure of cochlear implant or surgeries of the inner ear. In such cases, a smaller amount of GDNF protein product will be administered, for example, from about 1 mu g/ear to about 1 mg/ear in a single injection or in multiple injections. In cases where a chronic administration of the factor is needed, an Alzet mini-pump will be placed attached to a cannula the tip of which will be introduced into the middle or inner ear for a continuous release of the factor. GDNF can be also developed in a form of ear-drops which will penetrate the tympanic membrane of the Bulla. It is further contemplated that GDNF protein product be administered with an effective amount of a second therapeutic agent for the treatment of auditory neuron degeneration, together with BDNF and NT-3 as well as other factors or drugs used currently or in the future for the treatment of the various inner ear pathologies. A variety of pharmaceutical formulations and different delivery techniques are described in further detail below.

As used herein, the term "GDNF protein product" includes purified natural, synthetic or recombinant glial cell line-derived neurotrophic factor, biologically active GDNF variants (including insertion, substitution and deletion variants), and chemically modified derivatives thereof. Also included are GDNFs that are substantially homologous to the human GDNF having the amino acid sequence set forth in SEQ ID NO:1. GDNF protein products may exist as homodimers or heterodimers in their biologically active form.

The term "biologically active" as used herein means that the GDNF protein product demonstrates similar neurotrophic properties, but not necessarily all of the same properties, and not necessarily to the same degree, as the GDNF having the amino acid sequence set forth in SEQ ID NO: 1. The selection of the particular neurotrophic properties of interest depends upon the use for which the GDNF protein product is being administered.

The term "substantially homologous" as used herein means having a degree of homology to the GDNF having the amino acid sequence set forth in SEQ ID NO:1 that is preferably in excess of 70%, most preferably in excess of 80%, and even more preferably in excess of 90% or 95%. For example, the degree of homology between the rat and the human protein is about 93%, and it is contemplated that preferred mammalian GDNF will have a similarly high degree of homology. The percentage of homology as described herein is calculated as the percentage of amino acid residues found in the smaller of the two sequences which align with identical amino acid residues in the sequence being compared, when four gaps in a length of 100 amino acids may be introduced to assist in that alignment (as set forth by Dayhoff, in Atlas of Protein Sequence and Structure, Vol. 5, p. 124, National Biochemical Research Foundation, Washington, D.C. (1972), the disclosure of which is hereby incorporated by reference). In particular, Dayhoff describes that "[i]n practice, two related proteins may be aligned with the insertion of an average of 3 or 4 gaps in a length of 100 residues. About 20% of the aligned amino acids are identical. Under these conditions, the statistical conclusion of common ancestry can be drawn with great confidence. Common

ancestry may exist even though it cannot be proved from the comparison of two sequences. The use of additional evidence, such as the correspondence of the active sites, the comparisons of many related sequences with one new one, and the nature of the three-dimensional structures, will eventually permit the inference of relationships of even more remotely related structures." Also included as substantially homologous is any GDNF protein product which may be isolated by virtue of cross-reactivity with antibodies to the GDNF of SEQ ID NO:1 or whose genes may be isolated through hybridization with the gene or with segments of the gene encoding the GDNF of SEQ ID NO:1.

The GDNF protein products according to this invention may be isolated or generated by any means known to those skilled in the art. Exemplary methods for producing GDNF protein products useful in the present invention are described in U.S. application Ser. No. 08/182,183 filed May 23, 1994 and its parent applications; PCT Application No. PCT/US92/07888 filed Sep. 17, 1992, published as WO 93/06116 (Lin et al., Syntex-Synergen Neuroscience Joint Venture); European Patent Application No. 92921022.7, published as EP 610 254; and co-owned, co-pending U.S. application Ser. No. 08/535,681 filed Sep. 28, 1995 ("Truncated Glial Cell-Line Derived Neurotrophic Factor"), the disclosures of which are hereby incorporated by reference.

Naturally-occurring GDNF protein products may be isolated from mammalian neuronal cell preparations, or from a mammalian cell line secreting or expressing GDNF. For example, WO93/06116 describes the isolation of GDNF from serum-free growth conditioned medium of B49 glioblastoma cells. GDNF protein products may also be chemically synthesized by any means known to those skilled in the art. GDNF protein products are preferably produced via recombinant techniques because they are capable of achieving comparatively higher amounts of protein at greater purity. Recombinant GDNF protein product forms include glycosylated and non-glycosylated forms of the protein, and protein expressed in bacterial, mammalian or insect cell systems.

In general, recombinant techniques involve isolating the genes responsible for coding GDNF, cloning the gene in suitable vectors and cell types, modifying the gene if necessary to encode a desired variant, and expressing the gene in order to produce the GDNF protein product. Alternatively, a nucleotide sequence encoding the desired GDNF protein product may be chemically synthesized. It is contemplated that GDNF protein product may be expressed using nucleotide sequences which differ in codon usage due to the degeneracies of the genetic code or allelic variations. WO93/06116 describes the isolation and sequencing of a cDNA clone of the rat GDNF gene, and the isolation, sequencing and expression of a genomic DNA clone of the human GDNF gene. WO93/06116 also describes vectors, host cells, and culture growth conditions for the expression of GDNF protein product. Additional vectors suitable for the expression of GDNF protein product in E. coli are disclosed in published European Patent Application No. EP 0 423 980 ("Stem Cell Factor") published Apr. 24, 1991, the disclosure of which is hereby incorporated by reference. The DNA sequence of the gene coding for mature human GDNF and the amino acid sequence of the GDNF is shown in FIG. 19 (SEQ ID NO:5) of WO93/06116. FIG. 19 does not show the entire coding sequence for the pre-pro portion of GDNF, but the first 50 amino acids of human pre-pro GDNF are shown in FIG. 22 (SEQ ID NO:8) of WO93/06116.

Naturally-occurring GDNF is a disulfide-bonded dimer in its biologically active form. The material isolated after expression in a bacterial system is essentially biologically inactive, and exists as a monomer. Refolding is necessary to produce the biologically active disulfide-bonded dimer. Processes for the refolding and naturation of the GDNF expressed in bacterial systems are described in WO93/06116. Standard in vitro assays for the determination of GDNF activity are described in WO93/06116 and in co-owned, co-pending U.S. application Ser. No. 08/535,681 filed Sep. 28, 1995, and are hereby incorporated by reference.

A. GDNF variants

The term "GDNF variants" as used herein includes polypeptides in which amino acids have been deleted from ("deletion variants"), inserted into ("addition variants"), or substituted for ("substitution variants"), residues within the amino acid sequence of naturally-occurring GDNF. Such variants are prepared by introducing appropriate nucleotide changes into the DNA encoding the polypeptide or by in vitro chemical synthesis of the desired polypeptide. It will be appreciated by those skilled in the art that many combinations of deletions, insertions, and substitutions can be made provided that the final molecule possesses GDNF biological activity.

Mutagenesis techniques for the replacement, insertion or deletion of one or more selected amino acid residues are well known to one skilled in the art (e.g., U.S. Pat. No. 4,518,584, the disclosure of which is hereby incorporated by reference.) There are two principal variables in the construction of variants: the location of the mutation site and the nature of the mutation. In designing GDNF variants, the selection of the mutation site and nature of the mutation will depend on the GDNF characteristic(s) to be modified. The sites for mutation can be modified individually or in series, e.g., by (1) substituting first with conservative amino acid choices and then with more radical selections depending upon the results achieved, (2) deleting the target amino acid residue, or (3) inserting amino acid residues adjacent to the located site. Conservative changes in from 1 to 20 amino acids are preferred. Once the amino acid sequence of the desired GDNF protein product is determined, the nucleic acid sequence to be used in the expression of the protein is readily determined. N-terminal and C-terminal deletion variants may also be generated by proteolytic enzymes.

For GDNF deletion variants, deletions generally range from about 1 to 30 residues, more usually from about 1 to 10 residues, and typically from about 1 to 5 contiguous residues. N-terminal, C-terminal and internal intrasequence deletions are contemplated. Deletions may be introduced into regions of low homology with other TGF- beta super family members to modify the activity of GDNF. Deletions in areas of substantial homology with other TGF- beta super family sequences will be more likely to modify the GDNF biological activity more significantly. The number of consecutive deletions will be selected so as to preserve the tertiary structure of the GDNF protein product in the affected domain, e.g., cysteine crosslinking. Non-limiting examples of deletion variants include truncated GDNF protein products lacking from one to forty N-terminal amino acids of GDNF, or variants lacking the C-terminal residue of GDNF, or combinations thereof, as described in co-owned, co-pending U.S. application Ser. No. 08/535,681 filed Sep. 28, 1995, which is hereby incorporated by reference.

For GDNF addition variants, amino acid sequence additions typically include N-and/or C-terminal fusions ranging in length from one residue to polypeptides containing a hundred or more residues, as well as internal intrasequence additions of single or multiple amino acid residues. Internal additions may range generally from about 1 to 10 residues, more typically from about 1 to 5 residues, and usually from about 1 to 3 amino acid residues. Examples of N-terminal addition variants include GDNF with an N-terminal methionyl residue (an artifact of the direct expression of GDNF in bacterial recombinant cell culture), which is designated [Met< - 1>]GDNF, and fusion of a heterologous N-terminal signal sequence to the N-terminus of GDNF to facilitate the secretion of mature GDNF from recombinant host cells. Such signal sequences generally will be obtained from, and thus be homologous to, the intended host cell species. Additions may also include amino acid sequences derived from the sequence of other neurotrophic factors. A preferred GDNF protein product for use according to the present invention is the recombinant human [Met< - 1>]GDNF.

GDNF substitution variants have at least one amino acid residue of the GDNF amino acid sequence removed and a different residue inserted in its place. Such substitution variants include allelic variants, which are characterized by naturally-occurring nucleotide sequence changes in the species population that may or may not result in an amino acid change.

Examples of substitution variants (see, e.g., SEQ ID NO: 50) are disclosed in co-owned, co-pending U.S. application Ser. No. 08/535,681 filed Sep. 28, 1995, and are hereby incorporated by reference.

Specific mutations of the GDNF amino acid sequence may involve modifications to a glycosylation site (e.g., serine, threonine, or asparagine). The absence of glycosylation or only partial glycosylation results from amino acid substitution or deletion at any asparagine-linked glycosylation recognition site or at any site of the molecule that is modified by addition of an O-linked carbohydrate. An asparagine-linked glycosylation recognition site comprises a tripeptide sequence which is specifically recognized by appropriate cellular glycosylation enzymes. These tripeptide sequences are either Asn-Xaa-Thr or Asn-Xaa-Ser, where Xaa can be any amino acid other than Pro. A variety of amino acid substitutions or deletions at one or both of the first or third amino acid positions of a glycosylation recognition site (and/or amino acid deletion at the second position) result in non-glycosylation at the modified tripeptide sequence. Thus, the expression of appropriate altered nucleotide sequences produces variants which are not glycosylated at that site. Alternatively, the GDNF amino acid sequence may be modified to add glycosylation sites.

One method for identifying GDNF amino acid residues or regions for mutagenesis is called "alanine scanning mutagenesis" as described by Cunningham and Wells (Science, 244:1081-1085, 1989). In this method, an amino acid residue or group of target residues are identified (e.g., charged residues such as Arg, Asp, His, Lys, and Glu) and replaced by a neutral or negatively charged amino acid (most preferably alanine or polyalanine) to affect the interaction of the amino acids with the surrounding aqueous environment in or outside the cell. Those domains demonstrating functional sensitivity to the substitutions then are refined by introducing additional or alternate residues at the sites of substitution. Thus, the target site for introducing an amino acid sequence variation is determined, alanine scanning or random mutagenesis is conducted on the corresponding target codon or region of the DNA sequence, and the expressed GDNF variants are screened for the optimal combination of desired activity and degree of activity.

The sites of greatest interest for substitutional mutagenesis include sites where the amino acids found in GDNF proteins from various species are substantially different in terms of side-chain bulk, charge, and/or hydrophobicity. Other sites of interest are those in which particular residues of GDNF-like proteins, obtained from various species, are identical. Such positions are generally important for the biological activity of a protein. Initially, these sites are substituted in a relatively conservative manner. Such conservative substitutions are shown in Table 1 under the heading of preferred substitutions. If such substitutions result in a change in biological activity, then more substantial changes (exemplary substitutions) are introduced, and/or other additions or deletions may be made, and the resulting products screened for activity.

TABLE 1

Amino Acid Substitutions

Original Residue	Preferred Substitutions	Exemplary Substitutions
Ala (A)	Val	Val; Leu; Ile
Arg (R)	Lys	Lys; Gln; Asn
Asn (N)	Gln	Gln; His; Lys; Arg
Asp (D)	Glu	Glu
Cys (C)	Ser	Ser
Gln (Q)	Asn	Asn
Glu (E)	Asp	Asp

Gly (G)	Pro	Pro
His (H)	Arg	Asn; Gln; Lys; Arg
Ile (I)	Leu	Leu; Val; Met; Ala;
	*	Phe; norleucine
Leu (L)	Ile	norleucine; Ile; Val;
	*	Met; Ala; Phe
Lys (K)	Arg	Arg; Gln; Asn
Met (M)	Leu	Leu; Phe; Ile
Phe (F)	Leu	Leu; Val; Ile; Ala
Pro (P)	Gly	Gly
Ser (S)	Thr	Thr
Thr (T)	Ser	Ser
Trp (W)	Tyr	Tyr
Tyr (Y)	Phe	Trp; Phe; Thr; Ser
Val (V)	Leu	Ile; Leu; Met; Phe;
	*	Ala; norleucine

Conservative modifications to the amino acid sequence (and the corresponding modifications to the encoding nucleic acid sequences) are expected to produce GDNF protein products having functional and chemical characteristics similar to those of natural GDNF. In contrast, substantial modifications in the functional and/or chemical characteristics of GDNF protein products may be accomplished by selecting substitutions that differ significantly in their effect on maintaining (a) the structure of the polypeptide backbone in the area of the substitution, for example, as a sheet or helical conformation, (b) the charge or hydrophobicity of the molecule at the target site, or (c) the bulk of the side chain. Naturally occurring residues are divided into groups based on common side chain properties:

1) hydrophobic: norleucine, Met, Ala, Val, Leu, Ile;

2) neutral hydrophilic: Cys, Ser, Thr;

3) acidic: Asp, Glu;

4) basic: Asn, Gln, His, Lys, Arg;

5) residues that influence chain orientation: Gly, Pro; and

6) aromatic: Trp, Tyr, Phe.

Non-conservative substitutions may involve the exchange of a member of one of these classes for another. Such substituted residues may be introduced into regions of the GDNF protein that are homologous with other TGF- beta super family proteins, or into the non-homologous regions of the molecule.

B. GDNF Derivatives

Chemically modified derivatives of GDNF or GDNF variants may be prepared by one of skill in the art given the disclosures herein. The chemical moieties most suitable for derivatization include water soluble polymers. A water soluble polymer is desirable because the protein to

which it is attached does not precipitate in an aqueous environment, such as a physiological environment. Preferably, the polymer will be pharmaceutically acceptable for the preparation of a therapeutic product or composition. One skilled in the art will be able to select the desired polymer based on such considerations as whether the polymer/protein conjugate will be used therapeutically, and if so, the desired dosage, circulation time, resistance to proteolysis, and other considerations. The effectiveness of the derivatization may be ascertained by administering the derivative, in the desired form (i.e., by osmotic pump, or, more preferably, by injection or infusion, or, further formulated for oral, pulmonary or other delivery routes), and determining its effectiveness.

Suitable water soluble polymers include, but are not limited to, polyethylene glycol (PEG), copolymers of ethylene glycol/propylene glycol, carboxymethylcellulose, dextran, polyvinyl alcohol, polyvinyl pyrrolidone, poly-1,3-dioxolane, poly-1,3,6-trioxane, ethylene/maleic anhydride copolymer, polyaminoacids (either homopolymers or random copolymers), and dextran or poly(n-vinyl pyrrolidone) polyethylene glycol, propropylene glycol homopolymers, prolypropylene oxide/ethylene oxide co-polymers, polyoxyethylated polyols (e.g., glycerol), polyvinyl alcohol, and mixtures thereof. Polyethylene glycol propionaldehyde may have advantages in manufacturing due to its stability in water.

The polymer may be of any molecular weight, and may be branched or unbranched. For polyethylene glycol, the preferred molecular weight ranges from about 2 kDa to about 100 kDa for ease in handling and manufacturing (the term "about" indicating that in preparations of polyethylene glycol, some molecules will weigh more, some less, than the stated molecular weight). Other sizes may be used, depending on the desired therapeutic profile (e.g., the duration of sustained release desired, the effects, if any on biological activity, the ease in handling, the degree or lack of antigenicity and other known effects of polyethylene glycol on a therapeutic protein or variant).

The number of polymer molecules so attached may vary, and one skilled in the art will be able to ascertain the effect on function. One may mono-derivatize, or may provide for a di-, tri-, tetra- or some combination of derivatization, with the same or different chemical moieties (e.g., polymers, such as different weights of polyethylene glycols). The proportion of polymer molecules to protein (or peptide) molecules will vary, as will their concentrations in the reaction mixture. In general, the optimum ratio (in terms of efficiency of reaction in that there is no excess unreacted protein or polymer) will be determined by factors such as the desired degree of derivatization (e.g., mono-, di-, tri-, etc.), the molecular weight of the polymer selected, whether the polymer is branched or unbranched, and the reaction conditions.

The polyethylene glycol molecules (or other chemical moieties) should be attached to the protein with consideration of effects on functional or antigenic domains of the protein. There are a number of attachment methods available to those skilled in the art. See for example, EP 0 401 384, the disclosure of which is hereby incorporated by reference (coupling PEG to G-CSF), see also Malik et al., Exp. Hematol., 20:1028-1035, 1992 (reporting pegylation of GM-CSF using tresyl chloride). For example, polyethylene glycol may be covalently bound through amino acid residues via a reactive group, such as, a free amino or carboxyl group. Reactive groups are those to which an activated polyethylene glycol molecule may be bound. The amino acid residues having a free amino group may include lysine residues and the N-terminal amino acid residue. Those having a free carboxyl group may include aspartic acid residues, glutamic acid residues, and the C-terminal amino acid residue. Sulfhydryl groups may also be used as a reactive group for attaching the polyethylene glycol molecule(s). For therapeutic purposes, attachment at an amino group, such as attachment at the N-terminus or lysine group is preferred. Attachment at residues important for receptor binding should be avoided if receptor binding is desired.

One may specifically desire an N-terminal chemically modified protein. Using polyethylene

glycol as an illustration of the present compositions, one may select from a variety of polyethylene glycol molecules (by molecular weight, branching, etc.), the proportion of polyethylene glycol molecules to protein (or peptide) molecules in the reaction mix, the type of pegylation reaction to be performed, and the method of obtaining the selected N-terminally pegylated protein. The method of obtaining the N-terminally pegylated preparation (i.e., separating this moiety from other monopegylated moieties if necessary) may be by purification of the N-terminally pegylated material from a population of pegylated protein molecules. Selective N-terminal chemical modification may be accomplished by reductive alkylation which exploits differential reactivity of different types of primary amino groups (lysine versus the N-terminal) available for derivatization in a particular protein. Under the appropriate reaction conditions, substantially selective derivatization of the protein at the N-terminus with a carbonyl group containing polymer is achieved. For example, one may selectively N-terminally pegylate the protein by performing the reaction at a pH which allows one to take advantage of the pKa differences between the e-amino group of the lysine residues and that of the a-amino group of the N-terminal residue of the protein. By such selective derivatization, attachment of a water soluble polymer to a protein is controlled: the conjugation with the polymer takes place predominantly at the N-terminus of the protein and no significant modification of other reactive groups, such as the lysine side chain amino groups, occurs. Using reductive alkylation, the water soluble polymer may be of the type described above, and should have a single reactive aldehyde for coupling to the protein. Polyethylene glycol propionaldehyde, containing a single reactive aldehyde, may be used.

The present invention contemplates use of derivatives which are prokaryote-expressed GDNF, or variants thereof, linked to at least one polyethylene glycol molecule, as well as use of GDNF, or variants thereof, attached to one or more polyethylene glycol molecules via an acyl or alkyl linkage.

Pegylation may be carried out by any of the pegylation reactions known in the art. See, for example: Focus on Growth Factors, 3 (2):4-10, 1992; EP 0 154 316, the disclosure of which is hereby incorporated by reference; EP 0 401 384; and the other publications cited herein that relate to pegylation. The pegylation may be carried out via an acylation reaction or an alkylation reaction with a reactive polyethylene glycol molecule (or an analogous reactive water-soluble polymer).

Pegylation by acylation generally involves reacting an active ester derivative of polyethylene glycol with the GDNF protein or variant. Any known or subsequently discovered reactive PEG molecule may be used to carry out the pegylation of GDNF protein or variant. A preferred activated PEG ester is PEG esterified to N-hydroxysuccinimide. As used herein, "acylation" is contemplated to include without limitation the following types of linkages between the therapeutic protein and a water soluble polymer such as PEG: amide, carbamate, urethane, and the like. See Bioconjugate Chem., 5:133-140, 1994. Reaction conditions may be selected from any of those known in the pegylation art or those subsequently developed, but should avoid conditions of temperature, solvent, and pH that would inactivate the GDNF or variant to be modified.

Pegylation by acylation will generally result in a poly-pegylated GDNF protein or variant. Preferably, the connecting linkage will be an amide. Also preferably, the resulting product will be substantially only (e.g., > 95%) mono-, di- or tri-pegylated. However, some species with higher degrees of pegylation may be formed in amounts depending on the specific reaction conditions used. If desired, more purified pegylated species may be separated from the mixture, particularly unreacted species, by standard purification techniques, including, among others, dialysis, salting-out, ultrafiltration, ion-exchange chromatography, gel filtration chromatography and electrophoresis.

Pegylation by alkylation generally involves reacting a terminal aldehyde derivative of PEG with the GDNF protein or variant in the presence of a reducing agent. Pegylation by alkylation

can also result in poly-pegylated GDNF protein or variant. In addition, one can manipulate the reaction conditions to favor pegylation substantially only at the a-amino group of the N-terminus of the GDNF protein or variant (i.e., a mono-pegylated protein). In either case of monopegylation or polypegylation, the PEG groups are preferably attached to the protein via a -CH2-NH- group. With particular reference to the -CH2- group, this type of linkage is referred to herein as an "alkyl" linkage.

Derivatization via reductive alkylation to produce a monopegylated product exploits differential reactivity of different types of primary amino groups (lysine versus the N-terminal) available for derivatization. The reaction is performed at a pH which allows one to take advantage of the pKa differences between the e-amino groups of the lysine residues and that of the a-amino group of the N-terminal residue of the protein. By such selective derivatization, attachment of a water soluble polymer that contains a reactive group such as an aldehyde, to a protein is controlled: the conjugation with the polymer takes place predominantly at the N-terminus of the protein and no significant modification of other reactive groups, such as the lysine side chain amino groups, occurs. In one important aspect, the present invention contemplates use of a substantially homogeneous preparation of monopolymer/GDNF protein (or variant) conjugate molecules (meaning GDNF protein or variant to which a polymer molecule has been attached substantially only (i.e., > 95%) in a single location). More specifically, if polyethylene glycol is used, the present invention also encompasses use of pegylated GDNF protein or variant lacking possibly antigenic linking groups, and having the polyethylene glycol molecule directly coupled to the GDNF protein or variant.

Thus, it is contemplated that GDNF protein products to be used in accordance with the present invention may include pegylated GDNF protein or variants, wherein the PEG group(s) is (are) attached via acyl or alkyl groups. As discussed above, such products may be mono-pegylated or poly-pegylated (e.g., containing 2-6, and preferably 2-5, PEG groups). The PEG groups are generally attached to the protein at the a- or e-amino groups of amino acids, but it is also contemplated that the PEG groups could be attached to any amino group attached to the protein, which is sufficiently reactive to become attached to a PEG group under suitable reaction conditions.

The polymer molecules used in both the acylation and alkylation approaches may be selected from among water soluble polymers as described above. The polymer selected should be modified to have a single reactive group, such as an active ester for acylation or an aldehyde for alkylation, preferably, so that the degree of polymerization may be controlled as provided for in the present methods. An exemplary reactive PEG aldehyde is polyethylene glycol propionaldehyde, which is water stable, or mono C1-C10 alkoxy or aryloxy derivatives thereof (see, U.S. Pat. No. 5,252,714). The polymer may be branched or unbranched. For the acylation reactions, the polymer(s) selected should have a single reactive ester group. For the present reductive alkylation, the polymer(s) selected should have a single reactive aldehyde group. Generally, the water soluble polymer will not be selected from naturally-occurring glycosyl residues since these are usually made more conveniently by mammalian recombinant expression systems. The polymer may be of any molecular weight, and may be branched or unbranched.

A particularly preferred water-soluble polymer for use herein is polyethylene glycol. As used herein, polyethylene glycol is meant to encompass any of the forms of PEG that have been used to derivatize other proteins, such as mono- (C1-C10) alkoxy- or aryloxy-polyethylene glycol.

In general, chemical derivatization may be performed under any suitable condition used to react a biologically active substance with an activated polymer molecule. Methods for preparing pegylated GDNF protein or variant will generally comprise the steps of (a) reacting a GDNF protein or variant with polyethylene glycol (such as a reactive ester or aldehyde

derivative of PEG) under conditions whereby the protein becomes attached to one or more PEG groups, and (b) obtaining the reaction product(s). In general, the optimal reaction conditions for the acylation reactions will be determined case-by-case based on known parameters and the desired result. For example, the larger the ratio of PEG:protein, the greater the percentage of poly-pegylated product.

Reductive alkylation to produce a substantially homogeneous population of mono-polymer/GDNF protein (or variant) conjugate molecule will generally comprise the steps of: (a) reacting a GDNF protein or variant with a reactive PEG molecule under reductive alkylation conditions, at a pH suitable to permit selective modification of the a-amino group at the amino terminus of said GDNF protein or variant; and (b) obtaining the reaction product (s).

For a substantially homogeneous population of mono-polymer/GDNF protein (or variant) conjugate molecules, the reductive alkylation reaction conditions are those which permit the selective attachment of the water soluble polymer moiety to the N-terminus of GDNF protein or variant. Such reaction conditions generally provide for pKa differences between the lysine amino groups and the a-amino group at the N-terminus (the pKa being the pH at which 50% of the amino groups are protonated and 50% are not). The pH also affects the ratio of polymer to protein to be used. In general, if the pH is lower, a larger excess of polymer to protein will be desired (i.e., the less reactive the N-terminal a-amino group, the more polymer needed to achieve optimal conditions). If the pH is higher, the polymer:protein ratio need not be as large (i.e., more reactive groups are available, so fewer polymer molecules are needed). For purposes of the present invention, the pH will generally fall within the range of 3-9, preferably 3-6.

Another important consideration is the molecular weight of the polymer. In general, the higher the molecular weight of the polymer, the fewer polymer molecules may be attached to the protein. Similarly, branching of the polymer should be taken into account when optimizing these parameters. Generally, the higher the molecular weight (or the more branches) the higher the polymer:protein ratio. In general, for the pegylation reactions contemplated herein, the preferred average molecular weight is about 2 kDa to about 100 kDa. The preferred average molecular weight is about 5 kDa to about 50 kDa, particularly preferably about 12 kDa to about 25 kDa. The ratio of water-soluble polymer to GDNF protein or variant will generally range from 1:1 to 100:1, preferably (for polypegylation) 1:1 to 20:1 and (for monopegylation) 1:1 to 5:1.

Using the conditions indicated above, reductive alkylation will provide for selective attachment of the polymer to any GDNF protein or variant having an a-amino group at the amino terminus, and provide for a substantially homogenous preparation of monopolymer/GDNF protein (or variant) conjugate. The term "monopolymer/GDNF protein (or variant) conjugate" is used here to mean a composition comprised of a single polymer molecule attached to a molecule of GDNF protein or GDNF variant protein. The monopolymer/GDNF protein (or variant) conjugate preferably will have a polymer molecule located at the N-terminus, but not on lysine amino side groups. The preparation will preferably be greater than 90% monopolymer/GDNF protein (or variant) conjugate, and more preferably greater than 95% monopolymer/GDNF protein (or variant) conjugate, with the remainder of observable molecules being unreacted (i.e., protein lacking the polymer moiety).

For the present reductive alkylation, the reducing agent should be stable in aqueous solution and preferably be able to reduce only the Schiff base formed in the initial process of reductive alkylation. Preferred reducing agents may be selected from sodium borohydride, sodium cyanoborohydride, dimethylamine borane, trimethylamine borane and pyridine borane. A particularly preferred reducing agent is sodium cyanoborohydride. Other reaction parameters, such as solvent, reaction times, temperatures, etc., and means of purification of

products, can be determined case-by-case based on the published information relating to derivatization of proteins with water soluble polymers (see the publications cited herein).

C. GDNF Protein Product Pharmaceutical Compositions

GDNF protein product pharmaceutical compositions typically include a therapeutically effective amount of a GDNF protein product in admixture with one or more pharmaceutically and physiologically acceptable formulation materials. Suitable formulation materials include, but are not limited to, antioxidants, preservatives, coloring, flavoring and diluting agents, emulsifying agents, suspending agents, solvents, fillers, bulking agents, buffers, delivery vehicles, diluents, excipients and/or pharmaceutical adjuvants. For example, a suitable vehicle may be water for injection, physiological saline solution, or artificial perilymph, possibly supplemented with other materials common in compositions for parenteral administration. Neutral buffered saline or saline mixed with serum albumin are further exemplary vehicles.

The primary solvent in a vehicle may be either aqueous or non-aqueous in nature. In addition, the vehicle may contain other pharmaceutically-acceptable excipients for modifying or maintaining the pH, osmolarity, viscosity, clarity, color, sterility, stability, rate of dissolution, or odor of the formulation. Similarly, the vehicle may contain still other pharmaceutically-acceptable excipients for modifying or maintaining the rate of release of GDNF protein product, or for promoting the absorption or penetration of GDNF protein product across the tympanic membrane. Such excipients are those substances usually and customarily employed to formulate dosages for middle-ear administration in either unit dose or multi-dose form.

Once the therapeutic composition has been formulated, it may be stored in sterile vials as a solution, suspension, gel, emulsion, solid, or dehydrated or lyophilized powder. Such formulations may be stored either in a ready to use form or in a form, e.g., lyophilized, requiring reconstitution prior to administration.

The optimal pharmaceutical formulations will be determined by one skilled in the art depending upon considerations such as the route of administration and desired dosage. See for example, Remington's Pharmaceutical Sciences, 18th Ed. (1990, Mack Publishing Co., Easton, Pa. 18042) pages 1435-1712, the disclosure of which is hereby incorporated by reference. Such formulations may influence the physical state, stability, rate of in vivo release, and rate of in vivo clearance of the present GDNF proteins, variants and derivatives.

Other effective administration forms, such as middle-ear slow-release formulations, inhalant mists, or orally active formulations are also envisioned. For example, in a sustained release formulation, the GDNF protein product may be bound to or incorporated into particulate preparations of polymeric compounds (such as polylactic acid, polyglycolic acid, etc.) or liposomes. Hylauronic acid may also be used, and this may have the effect of promoting sustained duration in the circulation. The GDNF protein product pharmaceutical composition also may be formulated for middle-ear administration, e.g., by tympanic membrane infusion or injection, and may also include slow-release or sustained circulation formulations. Such middle-ear administered therapeutic compositions are typically in the form of a pyrogen-free, middle-ear acceptable aqueous solution comprising the GDNF protein product in a pharmaceutically acceptable vehicle. One preferred vehicle is sterile distilled water.

It is also contemplated that certain formulations containing GDNF protein product are to be administered orally. GDNF protein product which is administered in this fashion may be encapsulated and may be formulated with or without those carriers customarily used in the compounding of solid dosage forms. The capsule may be designed to release the active portion of the formulation at the point in the gastrointestinal tract when bioavailability is maximized and pre-systemic degradation is minimized. Additional excipients may be included

to facilitate absorption of GDNF protein product. Diluents, flavorings, low melting point waxes, vegetable oils, lubricants, suspending agents, tablet disintegrating agents, and binders may also be employed.

The formulation of topical ear preparations, including middle-ear solutions, suspensions and ointments is well known to those skilled in the art (see Remington's Pharmaceutical Sciences, 18th Edition, Chapter 86, pages 1581-1592, Mack Publishing Company, 1990). Other modes of administration are available, including injections to the middle ear, and methods and means for producing middle-ear preparations suitable for such modes of administration are also well known.

As used in this application, "middle-ear" refers to the space between the tympanic membrane and the inner ear. This location is external to all inner ear tissue and an invasive procedure might not be required to access this region if a formulation will be developed so that the GDNF will penetrate through the tympanic membrane. Otherwise, the material will be introduced to the middle ear by injection through the tympanic membrane or, in case repeated administrations are needed, a hole will be made in the tympanic membrane. Examples of such systems include inserts and "topically" applied drops, gels or ointments which may be used to deliver therapeutic material to these regions. An opening in the tymapanic membrane is a very frequent procedure done on a office-visit basis, in cases such as infections of the middle ear (usually in children). The opening closes spontaneously after a few days.

In the presently described use of GDNF protein product of the treatment of inner ear disease or injury it is also advantageous that a topically applied formulation include an agent to promote the penetration or transport of the therapeutic agent into the middle and inner ear. Such agents are known in the art. For example, Ke et al., U.S. Pat. No. 5,221,696 disclose the use of materials to enhance the penetration of ophthalmic preparations through the cornea.

Inner-ear systems are those systems which are suitable for use in any tissue compartment within, between or around the tissue layers of the inner-ear, such as the cochlea and vestibular organ. These locations include the different structures of the cochlea such as the stria vascularis, Reissner's membrane. organ of Corti, spiral ligament and the cochlear neurons. An invasive procedure might not be required to access those structures since it has been shown that protein do penetrate the membrane of the round window into the perylimph of the inner ear. A particularly suitable vehicle for introducing GDNF into the inner ear by penetration through the round window membrane is artificial perylimph. This solution consists of 10.00 mM D-glucose, 1,5 mM CaCl, 1.5 mM MgCl in a 1.0% solution of Dulbeco's phosphate-buffered saline in deionized water at 280-300 mOsm and pH of 7.2. Yet another preparation may involve the formulation of the GDNF protein product with an agent, such as injectable microspheres or liposomes into the middle ear, that provides for the slow or sustained release of the protein which may then be delivered as a depot injection. Other suitable means for the inner-ear introduction of GDNF protein product includes, implantable drug delivery devices or which contain the GDNF protein product, and a cochlear-implant with a tunnel through, so GDNF can be continuously delivered through it to the inner ear.

The ear-treatment preparations of the present invention, particularly topical preparations, may include other components, for example middle-ear acceptable preservatives, tonicity agents, cosolvents, complexing agents, buffering agents, antimicrobials, antioxidants and surfactants, as are well known in the art. For example, suitable tonicity enhancing agents include alkali metal halides (preferably sodium or potassium chloride), mannitol, sorbitol and the like. Sufficient tonicity enhancing agent is advantageously added so that the formulation to be instilled into the ear is compatible with the osmolarity of the endo- and perilymph. Suitable preservatives include, but are not limited to, benzalkonium chloride, thimerosal, phenethyl alcohol, methylparaben, propylparaben, chlorhexidine, sorbic acid and the like.

Hydrogen peroxide may also be used as preservative. Suitable cosolvents include, but are not limited to, glycerin, propylene glycol and polyethylene glycol. Suitable complexing agents include caffeine, polyvinylpyrrolidone, beta-cyclodextrin or hydroxypropyl-beta-cyclodextrin. The buffers can be conventional buffers such as borate, citrate, phosphate, bicarbonate, or Tris-HCl.

The formulation components are present in concentration that are acceptable to the middle or inner ear site of administration. For example, buffers are used to maintain the composition at physiological pH or at slightly lower pH, typically within a pH range of from about 5 to about 8.

Additional formulation components may include materials which provide for the prolonged the residence of the middle ear administered therapeutic agent so as to maximize the topical contact and promote absorbtion through the round window membrane. Suitable materials include polymers or gel forming materials which provide for increased viscosity of the middle-ear preparation. The suitability of the formulations of the instant invention for controlled release (e.g., sustained and prolonged delivery) of an inner-ear treating agent can be determined by various procedures known in the art. Yet another ear preparation may involve an effective quantity of GDNF protein product in a mixture with non-toxic middle-ear treatment acceptable excipients which are suitable for the manufacture of tablets. By dissolving the tablets in sterile water, or other appropriate vehicle, middle-ear treatment solutions can be prepared in unit dose form. Suitable excipients include, but are not limited to, inert diluents, such as calcium carbonate, sodium carbonate or bicarbonate, lactose, or calcium phosphate; or binding agents, such as starch, gelatin, or acacia.

Administration/Delivery of GDNF Protein Product

The GDNF protein product may be administered parenterally via a subcutaneous, intramuscular, intravenous, transpulmonary, transdermal, intrathecal or intracerebral route. For the treatment of inner-ear conditions, the GDNF protein product may be administered into the middle-ear (or directly into the inner-ear, especially in cases where invasive procedure is already in place), by topical application, inserts, injection, implants, cell therapy or gene therapy. For example, slow-releasing implants containing the neurotrophic factor embedded in a biodegradable polymer matrix can deliver GDNF protein product. GDNF protein product may be administered extracerebrally in a form that has been modified chemically or packaged so that it passes the blood-brain barrier, or it may be administered in connection with one or more agents capable of promoting penetration of GDNF protein product across the barrier. Similarly, the GDNF protein product may be administered in the middle or inner ear, or it may be administered on top of the tympanic membrane in connection with one or more agents capable of promoting penetration or transport of GDNF protein product across the membranes of the ear. The frequency of dosing will depend on the pharmacokinetic parameters of the GDNF protein product as formulated, and the route of administration.

The specific dose may be calculated according to considerations of body weight, body surface area or organ size. Further refinement of the calculations necessary to determine the appropriate dosage for treatment involving each of the above mentioned formulations is routinely made by those of ordinary skill in the art and is within the ambit of tasks routinely performed, especially in light of the dosage information and assays disclosed herein. Appropriate dosages may be ascertained through use of the established assays for determining dosages utilized in conjunction with appropriate dose-response data. It will be appreciated by those skilled in the art that the dosage used in inner-ear administered formulations will be minuscule as compared to that used in a systemic injection or oral administration.

The final dosage regimen involved in a method for treating the above-described conditions

will be determined by the attending physician, considering various factors which modify the action of drugs, e.g., the age, condition, body weight, sex and diet of the patient, the severity of any infection, time of administration and other clinical factors. As studies are conducted, further information will emerge regarding the appropriate dosage levels for the treatment of various diseases and conditions.

It is envisioned that the continuous administration or sustained delivery of GDNF may be advantageous for a given treatment. While continuous administration may be accomplished via a mechanical means, such as with an infusion pump, it is contemplated that other modes of continuous or near continuous administration may be practiced. For example, chemical derivatization or encapsulation may result in sustained release forms of the protein which have the effect of continuous presence, in predictable amounts, based on a determined dosage regimen. Thus, GDNF protein products include proteins derivatized or otherwise formulated to effectuate such continuous administration.

GDNF protein product cell therapy, e.g., middle- or inner ear implantation of cells producing GDNF protein product, is also contemplated. This embodiment would involve implanting into patients cells capable of synthesizing and secreting a biologically active form of GDNF protein product. Such GDNF protein product-producing cells may be cells that are natural producers of GDNF protein product (analogous to B49 glioblastoma cells) or may be recombinant cells whose ability to produce GDNF protein product has been augmented by transformation with a gene encoding the desired GDNF protein product in a vector suitable for promoting its expression and secretion. In order to minimize a potential immunological reaction in patients being administered GDNF protein product of a foreign species, it is preferred that the natural cells producing GDNF protein product be of human origin and produce human GDNF protein product. Likewise, it is preferred that the recombinant cells producing GDNF protein product be transformed with an expression vector containing a gene encoding a human GDNF protein product. Implanted cells may be encapsulated to avoid infiltration of surrounding tissue. Human or non-human animal cells may be implanted in patients in biocompatible, semipermeable polymeric enclosures or membranes that allow release of GDNF protein product, but that prevent destruction of the cells by the patient's immune system or by other detrimental factors from the surrounding tissue. Such an implant, for example, may be attached to the round-window membrane of the middle-ear to produce and release GDNF protein product directly into the perilymph.

It is also contemplated that the patient's own cells may be transformed ex vivo to produce GDNF protein product and would be directly implanted without encapsulation. For example, organ of Corti supporting cells may be retrieved, the cells cultured and transformed with an appropriate vector and transplanted back into the patient's inner ear where they would produce and release the desired GDNF protein or GDNF protein variant.

GDNF protein product gene therapy in vivo is also envisioned, by introducing the gene coding for GDNF protein product into targeted inner ear cells via local injection of a nucleic acid construct or other appropriate delivery vectors. (Hefti, J. Neurobiol., 25:1418-1435, 1994). For example, a nucleic acid sequence encoding a GDNF protein product may be contained in an adeno-associated virus vector or adenovirus vector for delivery to the inner ear cells. Alternative viral vectors include, but are not limited to, retrovirus, herpes simplex virus and papilloma virus vectors. Physical transfer, either in vivo or ex vivo as appropriate, may also be achieved by liposome-mediated transfer, direct injection (naked DNA), receptor-mediated transfer (ligand-DNA complex), electroporation, calcium phosphate precipitation or microparticle bombardment (gene gun).

The methodology for the membrane encapsulation of living cells is familiar to those of ordinary skill in the art, and the preparation of the encapsulated cells and their implantation in patients may be accomplished without undue experimentation. See, e.g., U.S. Pat. Nos. 4,892,538, 5,011,472, and 5,106,627, each of which is specifically incorporated herein by

reference. A system for encapsulating living cells is described in PCT Application WO 91/10425 of Aebischer et al., specifically incorporated herein by reference. See also, PCP Application WO 91/10470 of Aebischer et al., Winn et al., Exper. Neurol., 113:322-329, 1991, Aebischer et al., Exper. Neurol., 111:269-275, 1991; Tresco et al., ASAIO, 38:17-23, 1992, each of which is specifically incorporated herein by reference. Techniques for formulating a variety of other sustained-or controlled-delivery means, such as liposome carriers, bio-erodible particles or beads and depot injections, are also known to those skilled in the art.

It should be noted that the GDNF protein product formulations described herein may be used for veterinary as well as human applications and that the term "patient" should not be construed in a limiting manner. In the case of veterinary applications, the dosage ranges should be the same as specified above.

DETDESC:

Other aspects and advantages of the present invention will be understood upon consideration of the following illustrative examples. Example 1 addresses the effects of GDNF protein product administration on hair cells in a Cochlea explant culture system. Example 2 addresses the effects of GDNF protein product administration on spiral ganglion neurons, in a dissociated cell culture generated from cochlea. The results of the organ of Corti explant cultures studies and that of the adult rat spiral ganglion neuronal cultures demonstrated that GDNF protein product has neurotrophic and protective activity for the auditory neurons and the hair cells of the organ of Corti against ototoxins, which were not previously known to be GDNF-responsive.

EXAMPLES

Example 1

GDNF Protein Product Protects Cochlear Hair Cells Against Ototoxicity

MATERIALS

The materials used in the following Example were obtained as follows.

Organ of Corti dissecting solution:

Dulbecco's Phosphate Buffered Saline (PBS; 1 x , without calcium chloride, without magnesium chloride. Cat. #14190-136, Gibco BRL), containing 1.5 g/L D-Glucose (Dextrose. Cat. #15023-021, Gibco BRL).

Organ of Corti explant culture Medium

1. High glucose Dulbecco's Modified Eagle Medium (DMEM; 1 x , with L-glutamine, without Sodium Pyruvate. Cat. #11965-084, Gibco BRL)

2. 0.15 g/100 ml of D-Glucose (Dextrose. Cat. #15023-021, Gibco BRL)

3. 1% N-2 Supplement (100 x , Cat. #17502-030, Gibco BRL)

4. 100 Units/ml of Penicillin G, Potassium (Penicillin; Cat. #21840-020, Gibco BRL)

METHODS

Preparation of Medium

DMEM was supplemented with 1% N-2 supplement, and D-glucose was added to a final concentration of 1.5 g/L. Penicillin was added at 100 Units/ml. After mixing, the medium was filtered and kept at 4o C. The medium was prepared fresh just before use in order to minimize inter-experimental variations. Plastic pipettes and containers were used throughout to minimize protein adsorption.

GDNF Protein Product Solutions

Purified human recombinant GDNF protein products were prepared as 1 mg/ml solutions in D-PBS (phosphate buffered saline prepared with distilled water) containing five percent bovine serum albumin. The solutions were stored at - 85o C. in aliquots. Serial dilutions (0.1; 1; 10; 25; 50 ng/ml in normal culture medium) were prepared in 96 microplates. Ten microliters of ten-fold concentrated GDNF protein product solutions were added to Organ of Corti explant cultures medium containing ototoxins or not (control)(90 mu l). Control cultures received normal medium(10 mu l). The GDNF protein product treatments were initiated on day of plating. On the second day, media were exchanged into media containing the ototoxins alone, together with GDNF or without both (control).

Dissecting tools and culture dishes 1. The 4" and 5" dissecting forceps and 4" dissecting scissors were from Roboz Surgical, Washington, DC. 2. Falcon sterile 96-well microplates (Flat Bottom. Cat. #3072), tissue culture plastic ware and polypropylene centrifuge tubes were from Beckton-Dickinson, Lincoln Park, N.J.

Ototoxins and Related Reagents

1. Neomycin solution (Cat. #N1142, Sigma. St. Louis, Mo.), used at final concentration of 0.6 mM (A fresh solution was made each experiment by adding 90 mu l of 1 mg/ml neomycin and to 1410 mu l medium).

2. Cisplatin (Platinol-AQ. Cat. #NDC 0015-3220-22, Bristol-Myers Squibb Laboratories, Princeton, N.J.). Used at a final concentration of 35 mu g/ml (a fresh solution was prepared each experiment by adding 52.5 mu l of 1 mg/ml cisplatin to 1447.5 mu l medium)

3. Triton X-100 (t-Octylphenoxypoly-ethoxyethanol. Cat. #X-100, Sigma. St. Louis, Mo.)

4. Phalloidin (FITC Labeled. Cat. #P-5282, Sigma. St. Louis, Mo.)

5. Vectashield (Mounting Medium, Cat. #H-1000, Vector, Burlingame, Calif.)

Preparation of Rat Organ of Corti explant

Organ of Corti explants were obtained from P3-P4 Wistar rats. Rats were decapitated, the lower jaw was cut out and skin removed. The temporal bone was collected in dissection solution, the otic capsule exposed and the bony-cartilaginous cochlear capsule was carefully separated from the temporal bone. Freed cochlea were transferred to another Petri dish with dissection solution for further dissection. Intact organs of Corti were obtained by using a fine forceps to hold central VIII nerve tissue and remove it out, then the stria vascular membrane was carefully stripped off, starting from the apex or base. The organ of Corti then was then transferred to a 35-mm diameter Petri dish containing cold PBS supplemented with glucose and ready to be cultured.

Cochlea explant culture Procedure

Cochlea explants were cultured in uncoated 96 microplates. A single organ of Corti was placed in a well and was kept floating in the medium. Explants were kept in normal medium for 24 hours (90 mu l/well). GDNF protein solution (10 mu l) was added to the 'treated' cultures and 10 mu l medium was added to controls. After 24 hours of incubation, the media were changed and the explants were exposed to ototoxin-containing medium (90 mu l), with GDNF protein solution (10 mu l) or without (control). The cultures were incubated for an additional 3 days. The explants were then fixed with 4% paraformaldehyde in 0.1M D-PBS for 30 minutes at room temperature and processed for immunostaining.

FITC-phalloidin staining of hair cells

To identify and count hair cells in the organ of Corti, a direct immunostaining method was used to label the actin present naturally in the stereocilia bundles of the hair cells. The explants were washed three times with D-PBS (200 mu l per well) and permeabilized with 1% Triton X-100 in D-PBS for 15 minutes at room temperature. After three washes in D-PBS, the explants were incubated with FITC-labeled Phalloidin (1:60 from stock, 50 mu l/well) for 45 minutes at room temperature. The plates were covered with aluminum foil as the Phalloidin is light sensitive. After three more washes with D-PBS, the labeled explants were placed in a drop of glycerol on a microscope slide, covered with a glass coverslip and sealed with nail polish. The explants were observed under a Nikon Diaphot-300 inverted fluorescence microscope, using FITC filters and fluorescence optics.

Determination of hair cell number

For each experimental point, 2 to 4 cochlea were used. In each cochlea, the number of hair cells was counted in 2-3 sections, 175 mu m in length each. Only the sections in the middle turn of the cochlea were analyzed. Each experiment was repeated several times. The numbers of hair cells in control and cisplatin- or neomycin-treated cultures was generated from analyzing 40 cochlea per point.

RESULTS

Hair cells in the floating explant cultures did not die during the experiment period of four days. Thus, the number of phalloidin-stained cells present at the end of the 4 days experiment period, in the absence of ototoxins and treatments, was 105.4 +/- 6.9 (n = 28). Ototoxins added to the explants on the second day post plating caused significant loss in hair cell number found after 4 days in vitro. Exposure to 35 mu g/ml cisplatin 24 hours after plating caused a loss of about 80 percent of the hair cells: only 21.2% +/- 6.0 (n = 40) of the initial number of hair cells survived (Table 2) and after exposure to 0.8 mM neomycin, only 7.4% +/- 4.7 (n = 43) of the hair cells survived (Table 2).

TABLE 2

Effect of GDNF on cochlear hair cells exposed to cisplatin

Hair Cell Survival (% of untreated)

Treatment	GDNF added at time of plating	GDNF added 24 hours after plating
Cisplatin alone (35 mu g/ml)	21.3 +/- 4.0 (n = 30)	21.3 +/- 4.0 (n = 30)
Cisplatin + GDNF, 0.05 ng/ml	35.2 +/- 5.4 (n = 5)*	ND
Cisplatin + GDNF, 0.1 ng/ml	39.6 +/- 10.5 (n = 17)*	37.8 +/- 11.8 (n = 5)*
Cisplatin + GDNF, 1 ng/ml	46.7 +/- 10.8 (n = 20)*	51.0 +/- 8.0 (n = 4)*

Cisplatin + GDNF, 10 ng/ml	46.7 +/- 7.7 (n = 16)*	49.7 + 4.6 (n = 5)*
Cisplatin + GDNF, 25 ng/ml	ND	45.0 +/- 12.0 (n = 3)*
Cisplatin + GDNF, 50 ng/ml	46.8 +/- 10.5 (n = 13)*	ND

nGDNF was introduced to the explant cultures either on the day of plating or 24 hours after plating. Cisplatin (35 mu g/ml) was added 24 hours after plating, and the cultures were incubated for an additional 3 days. The hair cells were stained with FITC-phalloidin. The number of hair cells was counted in the middle turn of the cochlea in 2-3 sections of 175 mu m each. The results are expressed as the percentage of hair cells present in untreated cultures after 4 days in vitro (105.4 +/- 6.9; n = 28). Each number is the mean +/- SD of n cochleas. -

n*Significantly different from cisplatin alone at $p < 0.05$ (t-Test) -

nND: not determined -

There was a marked difference in the morphology of the organs of Corti between these two treatments: while the treatment with neomycin resulted in almost complete loss of hair cells, those that were spared were still organized in the typical four row structure (3 rows of outer hair cells and one row of inner hair cells). Cisplatin treatment, on the other hand, caused a marked disruption of the four-row-structure and the surviving cells were randomly located.

In cultures that received GDNF at the time of plating (pretreatment), a significant number of hair cells survived the 3-day exposure to ototoxins (from day 2 to day 4). In cultures exposed to cisplatin, treatment with GDNF concentrations as low as 0.05 ng/ml caused an increase in surviving hair cells from 21% (untreated cultures) to 35%. Maximal protective activity was reached with 0.1 ng/ml GDNF (41% survival) (Table 3). In cultures exposed to neomycin, GDNF at 0.1 ng/ml increased the number of hair cells from 7% to 22%; maximal GDNF activity (37% survival) was seen with 10 ng/ml GDNF (Table 3). GDNF treatment preserved the four-raw morphology in neomycin-treated cultures, but did not prevent its disruption by cisplatin.

To test further the ability of GDNF to rescue hair cells from ototoxicity, a set of experiments was performed in which GDNF was added 24 hours after plating, at the same time as the cultures were exposed to the toxins (co-treatment). The results indicate that under this experimental paradigm GDNF is capable of rescuing hair cells to the same extent as when given prior to the exposure to the toxins (Tables 2 and 3)

TABLE 3

Effect of GDNF on cochlear hair cells exposed to neomycin

	Hair Cell Survival (% of untreated)	
Treatment	GDNF added at time of plating	GDNF added 24 hr after plating
Neomycin alone(0.8 mM)	7.1 +/- 4.2 (n = 42)	7.1 + 4.2 (n = 42)
Neomycin + GDNF, 0.05 ng/ml	19.5 +/- 7.5 (n = 6)*	23.0 +/- 6.2 (n = 3)*

Neomycin + GDNF, 0.1 ng/ml	22.0 +/- 4.1 (n = 13) *	27.0 +/- 14.7 (n = 3)*
Neomycin + GDNF, 1 ng/ml	28.2 +/- 6.1 (n = 19) *	26.2 +/- 6.4. (n = 4)*
Neomycin + GDNF, 10 ng/ml	37.4 +/- 4.8 (n = 11) *	ND
Neomycin + GDNF, 50 ng/ml	34.4 + 5.3 (n = 7)*	ND

nGDNF was introduced to the explant cultures either on the day of plating (pretreatment) or 24 hours af ter plating (co-treatment). Neomycin (0.8M) was added 24 hours after plating, and the cultures were incubated for an additional 3 days. The hair cells were stained with FITC-phalloidin. The number of hair cells was counted in the middle turn of the cochlea in 2-3 sections of 175 mu m each. The results are expressed as the percentage of hair cells present in untreated cultures after 4 days in vitro (105.4 +/- 6.9; n = 28). Each number is the mean +/- SD of n cochleas. -

n*Significantly different from neomycin alone at $p < 0.05$ (t-Test) -

nND: not determined -

Example 2

GDNF Protein Product Promotes Survival of Inner Ear Auditory Neurons (Spiral Ganglion Neurons) and Protects Them Against Ototoxins

MATERIALS

The materials used in the following Example were obtained as follows.

Cell Culture Media

High glucose Dulbecco's Modified Eagle's Medium (DMEM; #11965-092), Ham's F12 medium (F12;#11765-021), B27 medium supplement (#17504-010), penicillin/streptomycin (#15070-014), L-glutamine (#25030-016), Dulbecco's phosphate-buffered saline (D-PBS; #14190-052), mouse laminin (#23017-015), bovine serum albumin and fractionV (#110-18-017) were all from GIBCO/BRL, Grand Island, N.Y. Heat-inactivated horse serum was from HyClone, Logan, Utah. Poly-L-ornithine hydrobromide (P-3655), bovine insulin (I-5500), human transferrin (T-2252), putrescine (P-6024), progesterone (P-6149) and sodium selenite (S-9133) were all from Sigma Chemical Company, Saint-Louis, Mo. Papain, deoxyribonuclease I (DNAase) and ovalbumin (Papain dissociation system) were from Worthington Biochemicals, Freehold, N.J. Falcon sterile 96-well microplates (#3072), tissue culture plastic ware and polypropylene centrifuge tubes were from Beckton-Dickinson, Oxnard, Calif. Nitex 20 mu m nylon mesh (#460) was from Tetko, Elmsford, N.Y. The 4" dissecting forceps and 4" dissecting scissors were from Roboz Surgical, Washington, D.C.

Antibodies and Related Reagents

Neuronal Specific Enolase (NSE) rabbit polyclonal antibody, was from Chemicon (#AB951), biotinylated goat anti-rabbit IgG (#BA-1000) and peroxidase-conjugated avidin/biotin complex (ABC Elite; kit PK-6100) were from Vector Laboratories, Burlingame, Calif. 3', 3'-diaminobenzidine was from Cappel Laboratories, West Chester, Pa. Superblock blocking buffer in PBS (#37515) was from Pierce, Rockford, Ill. Triton X-100 (X100), Nonidet P-40 (N6507) and hydrogen peroxide (30%, v/v; H1009) were from Sigma. All other reagents were obtained from Sigma Chemical Company (Saint-Louis, Mo.), unless otherwise specified.

Ototoxins

Cisplatin (Plàtinol-AQ, #NDC 0015-3220-22) was from Bristol-Myers-Squibb, Princeton, N.J., sodium salicylate was from J. T.Baker, Phillipsburg, N.J. (#3872-01) and neomycin was from Sigma (#N1142).

METHODS

Preparation of Media

A basal medium was prepared as a 1:1 mixture of DMEM and F12 medium, and was supplemented with B27 medium supplement added as a 50-fold concentrated stock solution. The B27 medium supplement consists of biotin, L-carnitine, corticosterone, ethanolamine, D (+) - galactose, reduced glutathione, linoleic acid, linolenic acid, progesterone, putrescine, retinyl acetate, selenium, T3 (triodo-1-thyronine, DL-alpha-tocopherol; vitamin E), DL-alpha-tocopherol acetate, bovine serum albumin, catalase, insulin, superoxide dismutase and transferrin. L-glutamine was added at a final concentration of about 2 mM, penicillin at about 100 IU/l, and streptomycin at about 100 mg/l. Heat-inactivated horse serum was added to a final concentration of about 2.5 percent, D-glucose was added to a final concentration of about 5 g/l, HEPES buffering agent was added to a final concentration of about 20 mM, bovine insulin was added to a final concentration of about 2.5 mg/ml, and human transferrin was added to a final concentration of about 0.1 mg/ml. After mixing, the pH was adjusted to about 7.3 and the medium was kept at 40o C. The media were prepared fresh just before use in order to minimize inter-experimental variations. Plastic pipettes and containers were used throughout to minimize protein adsorption.

GDNF Protein Product Solutions

Purified human recombinant GDNF protein products were prepared as 1 mg/ml solutions in D-PBS (phosphate-buffered saline prepared with distilled water) containing five percent bovine serum albumin. The solutions were stored at - 85o C. in aliquots. Serial dilutions were prepared in 96-well microplates. Ten microliters of ten-fold concentrated GDNF protein product solutions were added to cell cultures containing culture medium (90 mu l). Control cultures received D-PBS with 5 percent albumin (10 mu l). The GDNF protein product treatments were added to the cultures one hour after cells were seeded or 24 hours later, alone or together with the ototoxins.

Ototoxins preparations

Neomycin was added straight from the stock solution (about 10<31 3> M) at 10 mu l per well to result in a final concentration of about 10<31 4> M. Cisplatin was diluted with culture medium from the stock solution (1 mg/ml) to a solution of 20 mu g/ml and added at 10 mu l per well, to result in a final concentration of 2 mu g/ml. Sodium salicylate was prepared from powder to a stock solution of 1M in PBS and further diluted in the culture medium to 100 mM, which resulted in a 10 mM final concentration when added at 10 mu l/well to the culture.

Culture Substratum

To encourage optimal attachment of spiral ganglion cells on substratum and neurite outgrowth, the microtiter plate surfaces (the culture substratum) were modified by sequential coating with poly-L-ornithine followed by laminin in accordance with the following procedure. The plate surfaces were completely covered with a 0.1 mg/ml sterile solution of polyornithine in 0.1M boric acid (pH 8.4) for at least one hour at room temperature, followed by a sterile wash with Super-Q water. The water wash was then aspirated and a 10 mu g/ml solution of mouse laminin in PBS was added and incubated at 37o C. for two hours. These procedures were conducted just before using the plates in order to ensure reproducibility of the results.

Preparation of Rat Spiral Ganglion Cell Cultures

Three- to four-week-old Wistar rats (obtained from Jackson Laboratories, Bar Harbor, Me.) were injected with an overdose of the following solution (ketamine (100 mg/ml); Xylazine (20 mg/ml) and Acopromazine Maleate 910 mg/ml) at 3:3:1 proportions), killed by decapitation and the temporal bone with the cochlea were dissected out and transferred sterilely into PBS with 1.5 g/L glucose on ice. A maximum of 30 cochlea were processed per experiment. The cochlea were opened and the organ of Corti with the bony modiolus was collected into 50 ml sterile tube containing 5 ml dissociation medium (120 units papain and 2000 units DNAase in HBSS). The tissue was incubated for 30 minutes at about 37o C. on a rotary platform shaker at about 200 rpm and then the dissociation solution was replaced with a fresh one and the incubation resumed for another 30 min. The cells were then dispersed by trituration through fire-polished Pasteur pipettes, sieved through a 40 mu m Nitex nylon mesh to discard undissociated tissue, and centrifuged for five minutes at 200 x g using an IEC clinical centrifuge. The resulting cell pellet was resuspended into HBSS containing ovalbumin and about 500 units DNAase, layered on top of a four percent ovalbumin solution (in HBSS) and centrifuged for about 6 minutes at 500 x g. The final pellet was resuspended into about 6 ml of the culturing medium and seeded at 90 mu l/well in the precoated plates.

Immunohistochemistry of spiral ganglion cells

Spiral ganglion neurons were identified by immunohistochemical staining for neuronal specific enolase (NSE). Cultures of spiral ganglion cells were fixed for about 10 minutes at room temperature with eight percent paraformaldehyde in D-PBS, pH 7.4, added at 100 mu l/well to the culture medium and then replaced by 100 mu l of four percent paraformaldehyde for additional 10 minutes, followed by three washes in D-PBS (200 mu l per 6-mm well). The fixed cultures were then incubated in Superblock blocking buffer in PBS, containing one percent Nonidet P-40 to increase the penetration of the antibody. The rabbit polyclonal anti-NSE antibodies (Chemicon) were then applied at a dilution of 1:6000 in the same buffer, and the cultures were incubated for two hours at 37o C. on a rotary shaker. After three washes with D-PBS, the spiral ganglion cell-bound antibodies were detected using goat-anti-rabbit biotinylated IgG (Vectastain kit from Vector Laboratories, Burlingame, Calif.) at about a 1:300 dilution. The secondary antibody was incubated with the cells for about one hour at 37o C., the cells were then washed three times with D-PBS. The secondary antibody was then labeled with an avidin-biotin-peroxidase complex diluted at 1:300, and the cells were incubated for about 60 minutes at 37o C. After three more washes with D-PBS, the labeled cell cultures were reacted for 5 minutes in a solution of 0.1M Tris-HCl, pH 7.4, containing 0.04% 3', 3'-diaminobenzidine-(HCl)4, 0.06 percent NiCl2 and 0.02 percent hydrogen peroxide.

Determining spiral Ganglion Cell Survival

After various times in culture (24 hours, 3 days and 4 days), rat spiral ganglion cell cultures were fixed, processed and immunostained for NSE as described above, and the cultures were

then examined with bright-light optics at 200 x magnification. All the NSE-positive neurons present in a 6-mm well were counted. Viable spiral ganglion cells were characterized as having a round body of size ranging from 15-40 mu m and bearing neuritic processes. Spiral ganglion cells showing signs of degeneration, such as having irregular, vacuolated perikarya or fragmented neurites, were excluded from the counts (most of the degenerating spiral ganglion cells, however, detached from the culture substratum). Cell numbers were expressed either as cells/6-mm well or as the fold-change relative to control cell density.

RESULTS

Cultures of rat spiral ganglion neurons were used to demonstrate the effect of GDNF protein product on survival and protection against ototoxins. The spiral ganglion cells were obtained from three to four -week old rat cochlea. The dissociated cells were then seeded into polyornithine-laminin-coated microplates at a density of about 1 cochlea per 2 wells in DMEM/F12 supplemented with B27 medium supplement, 2.5 percent heat-inactivated horse serum, D-glucose, HEPES, insulin and transferrin. The cultures consisted of a mixture of neurons and non-neuronal cells. However, the only neurons present were spiral ganglion neurons and were identified by the presence of NSE immunoreactivity. At the concentration seeded(1 dissociated organ of Corti into 2 wells), the number of NSE-positive cells per well 24 hours after seeding was 127 +/- 17 (n = 7) under control conditions (no added treatments). At the end of the experiment, 4 days after seeding, the number of the neurons per well was reduced to 64 +/- 4.7, which represents 50.5% +/- 3.7 of the number present after 24 hours in vitro, under control conditions.

The effect of GDNF protein product administration was assessed on the survival and morphological maturation of cultured rat spiral ganglion neurons, as well as on their ability to resist the toxic effect of a known ototoxin such as cisplatin. Cultures of spiral ganglion cells were treated 24 hours after seeding with human recombinant GDNF protein product (ranging from 50 ng/ml to 0.1 ng/ml) alone, or in combination with cisplatin (35 mu g/ml). Twenty four hours after seeding, there was no difference in the number of auditory neurons between control cultures and those treated with GDNF at 1 ng/ml and 10 ng/ml (127 +/- 17; 126 +/- 24 and 125 +/- 19 neurons/well respectively). After an additional period of 3 days, treatment with GDNF at a concentration of 1 ng/ml did not result in a significant increase in neuronal cell number. There was however, a marked trophic effect: the neuronal soma were larger and fibers longer and more elaborate than in control cultures. In cultures treated with 10 ng/ml GDNF, about 72% of the neurons present after 24 hours survived, representing a 44% increase over control cultures (Table 4). The trophic effect was even stronger than in cultures treated with 1 ng/ml GDNF.

TABLE 4

Effect of GDNF on spiral ganglion neuron survival

Treatments	Spiral Ganglion Neuron Survival (% of initial number after 24 hours)	
	None	Cisplatin (5 mu g/ml)
None	48.5 +/- 4.5 (n = 9)	6.1 +/- 1.2 (n = 3)
GDNF, 10 ng/ml	71.6 + 7.4** (n = 5)	32.8 +/- 1.0* (n = 3)

nGDNF and cisplatin were added to dissociated spiral ganglion neuron cultures 24 hours after plating. The cultures were incubated for an additional 3 days and the number of neurons was determined by counting NSE-immunoreactive cells. Neuronal cell numbers are expressed as

the percentage of neurons present after 24 hours in vitro. Each result is the mean +/- SD of n cultures. -

n*Significantly different from cisplatin alone at $p < 0.05$ (t-Test) -

n**Significantly different from untreated control at $p < 0.05$ (t-Test) -

GDNF also protected spiral ganglion neurons from cisplatin toxicity (Table 4). Exposure of cultures to 5 mu g/ml cisplatin 24 hours after seeding resulted in the loss of about 94% of the initial number (at 24 hours) of the neurons after 4 days in culture. When GDNF was added together with the cisplatin, the number of neurons found after 4 days was significantly higher. This protective effect of GDNF was dose-dependent: 20.1 +/- 5.1; 27.5 +/- 3.2 and 32.8 +/- 1.0 of the neurons present at the beginning of the toxic treatment were found with GDNF concentrations of 1 ng/ml, 10 ng/ml and 25 ng/ml respectively (data not shown). These results indicate that about 63 percent of the neurons that respond to GDNF (about 44% of the spiral ganglion neuron population) can also be protected against cisplatin toxicity.

Numerous modifications and variations in the practice of the invention are expected to occur to those skilled in the art upon consideration of the foregoing description of the presently preferred embodiments thereof.

```
SEQUENCE LISTING
- - (1) GENERAL INFORMATION:
- (iii) NUMBER OF SEQUENCES: 2
- - (2) INFORMATION FOR SEQ ID NO:1:
- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 134 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: protein
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:
- SerProAspLysGlnMetAlaValLeuProArgArgGluArgAsnArg
151015
- GlnAlaAlaAlaAlaAsnProGluAsnSerArgGlyLysGlyArgArg
202530
- GlyGlnArgGlyLysAsnArgGlyCysValLeuThrAlaIleHisLeu
354045
- AsnValThrAspLeuGlyLeuGlyTyrGluThrLysGluGluLeuIle
505560
- PheArgTyrCysSerGlySerCysAspAlaAlaGluThrThrTyrAsp
65707580
- LysIleLeuLysAsnLeuSerArgAsnArgArgLeuValSerAspLys
859095
- ValGlyGlnAlaCysCysArgProIleAlaPheAspAspAspLeuSer
100105110
- PheLeuAspAspAsnLeuValTyrHisIleLeuArgLysHisSerAla
115120125
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- LysArgCysGlyCysIle
130
- - (2) INFORMATION FOR SEQ ID NO:2:
- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 402 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: other nucleic acid
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:
- TCACCAGATAAACAAATGGCAGTGCTTCCTAGAAGAGAGCGGAATCGGCAGGCTGCAGCT60
- GCCAACCCAGAGAATTCCAGAGGAAAAGGTCGGAGAGGCCAGAGGGGCAAAAACCGGGGT120
- TGTGTCTTAACTGCAATACATTTAAATGTCACTGACTTGGGTCTGGGCTATGAAACCAAG180
- GAGGAACTGATTTTTAGGTACTGCAGCGGCTCTTGCGATGCAGCTGAGACAACGTACGAC240
- AAAATATTGAAAAACTTATCCAGAAATAGAAGGCTGGTGAGTGACAAAGTAGGGCAGGCA300
- TGTTGCAGACCCATCGCCTTTGATGATGACCTGTCGTTTTTAGATGATAACCTGGTTTAC360
- CATATTCTAAGAAAGCATTCCGCTAAAAGGTGTGGATGTATC402
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CLAIMS: What is claimed is:

[*1] 1. A method for treating injury or degeneration of cells of the inner ear, comprising administering a glial cell line-derived neurotrophic factor (GDNF) protein product comprising an amino acid sequence set forth in SEQ ID NO:1, wherein said GDNF protein product promotes the survival or function of cochlear hair cells and auditory neurons of the inner ear.

[*2] 2. The method of claim 1, wherein said auditory neurons are spiral ganglion neurons.

[*3] 3. The method of claim 1, wherein the GDNF protein product further comprises an amino terminal methionine.

[*4] 4. The method of claim 1, wherein the GDNF protein product is administered at a dose of about 1 mu g/kg/day to about 100 mg/kg/day.

[*5] 5. A method for treating injury or degeneration of cells of the inner ear comprising administering a glial cell line-derived neurotrophic factor (GDNF) protein product comprising an amino acid sequence which is in excess of 70% identical to an amino acid sequence set forth in SEQ ID NO:1 when up to four gaps in a length of 100 amino acids may be introduced to assist in that alignment, wherein said GDNF protein product promotes the survival or function of cochlear hair cells and auditory neurons of the inner ear.

[*6] 6. The method of claim 5, wherein the GDNF protein product further comprises an amino terminal methionine.

[*7] 7. The method of claim 5, wherein the GDNF protein product is administered at a dose of about 1 mu g/kg/day to about 100 mg/kg/day.

[*8] 8. The method of claim 5, wherein said auditory neurons are spiral ganglion neurons.

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